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Original Article/Orijinal Makale

PRELIMINARY CHARACTERIZATION OF CRUDE INULINASE ACTIVITY OF *ASPERGILLUS WENTII*

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Abstract: This study aims to perform a partial characterization of the crude enzymatic extracts of *Aspergillus wentii* inulinase produced by using the Jerusalem artichoke as the only carbon source in a production medium, which is a new source for obtaining inulinase. The screening medium that contained inulin as the only carbon source used by Derycke and Wandamme (1984) was redesigned to include Jerusalem artichoke powder instead of inulin and was used for the inulinase production of *A. wentii*. The crude inulinase produced by *A.wentii* was preliminarily characterized for its activity and stability. Optimum pH, temperature and incubation times for the crude enzymatic extract were determined as pH 6.0, 35°C and 10 min, respectively. The K_m and the V_{max} values were calculated as approximately 1×10^{-4} M, and 6.134 mmol/ml/min for *A. wentii* inulinase, respectively. The enzyme activity was observed to be stable at the intervals of pH 3.0-6.0 (96.8%), to maintain its activity until 50°C, and to have 62.3% relative activity at 80°C. The results confirm that *A. wentii* inulinase can be an enzyme with industrial treatments due to its relationship with its substrate and its good pH stability and thermal stability. *A. wentii* inulinase can be a new and usable source for preparation of pure fructose from inulin-containing agricultural products.

Key words: *Aspergillus wentii*, Inulinase, Jerusalem artichoke, partial characterization.

Aspergillus wentii Kaba İnulinaz Aktivitesinin Ön Karakterizasyonu

Özet: İnülinazlar fonksiyonel besin maddeleri olarak kullanılan inülin ve fruktooligosakkaritlerin enzimatik hidrolizi ile fruktoz şurubu üretiminde kullanılabilen önemli bir enzim grubudur. Bu çalışmada inulinaz eldesi için yeni bir kaynak olan ve üretim ortamında tek karbon kaynağı olarak yer elması kullanılarak üretilen *Aspergillus wentii* inulinazının kaba enzimatik ekstraktlarının kısmi karakterizasyonunun gerçekleştirilmesi hedeflendi. Derycke ve Wandamme (1984)'ın kullandığı, karbon kaynağı olarak sadece inülin içeren tarama medyumu, inülin yerine yer elması tozu kullanılarak yeniden düzenlendi ve *A. wentii*'nin inülinaz üretimi için kullanıldı. *A. wentii* inulinazı'nın aktivite ve stabilite açısından kısmi karakterizasyonu gerçekleştirildi. Kaba enzimatik ekstrakt için optimum pH, sıcaklık ve inkübasyon süresi sırası ile pH 6,0, 35˚C ve 10 dk olarak belirlendi. *A. wentii* inülinazı için K_m değeri yaklaşık 1×10^{-4} M, V_{max} değeri ise 6,134 mmol/ml/dk olarak hesaplandı. Enzimin pH 3,0-6,0 aralığında aktivitesinin kararlı olduğu (%96,8) ve 50˚C ye kadar aktivitesini koruduğu ve 80˚C'de %62,3 bağıl aktiviteye sahip olduğu görüldü. Elde edilen sonuçlar *A. wentii* inulinazının subtratı ile olan ilişkisi, iyi bir pH ve termal kararlılığa sahip olması göz önüne alındığında endüstriyel olarak kullanılabilir bir enzim olduğunu doğrulamaktadır. *A. wenti* inulinazı inulin içeren tarımsal ürünlerden saf fruktoz şurubu hazırlanması için yeni ve uygun bir kaynak olabilir.

Anahtar kelimeler: Aspergillus wentii, İnulinaz, yerelması, kısmi karakterizasyonu

Introduction

Microbial enzymes which are used in almost every industrial field are preferred over plant- or animal-based enzymes because they have higher catalytic activity, do not produce by-products, are more stable and cheaper and can be obtained at higher amounts (Kıran et al. 2006). Since most enzymes that are currently used in food drink and pharmaceutical industries are of microbial origin, the use of microorganisms in production of industrial enzymes has intensified (Kango & Jain 2011).

Natural polysaccharide is widely used in industry today and new polysaccharide sources are being sought. Therefore, there is a growing interest in the production of extracellular polysaccharides by fermentation. The sugar industry encounters competition with fructose syrups, which are widely used as low-cost alternative sweeteners (Sanal et al. 2005). Fructose syrup is a monosaccharide sugar obtained by converting corn starch into glucose and by the enzymatic isomerization of this glucose. The sweetness degree of fructose syrup is similar to the invert sugar group. Fructose syrups prevent products from drying

due to their moisture retention characteristic. They can be confidently used to flavor food due to their flavor-enhancing properties (Artık et al. 2011). The method used to obtain fructose syrup from starch is quite an expensive one (Gill et al. 2003). Fructose production from the enzymatic hydrolysis of inulin is a promising process that has drawn much attention in recent years, as this method yields higher fructose concentration than conventional glucose isomerization (Ricca et al. 2010). Ninety-five percent of fructose production is achieved by the hydrolysis of inulin through inulinase enzyme (Sguarezi et al. 2009). The enzymes preferred in the preparation of fructose syrup are produced by yeasts and filamentous fungi, and inulinases produced by microorganisms growing in an inulin environment have therefore drawn the attention of researchers (Gupta et al. 1998). In this study, *Aspergillus wentii*, which was found to synthesize inulinase (EC.3.2.1.7.) by being produced in a screening medium containing Jerusalem artichoke as the inulin source, was used as inulinase source. We obtained the inulinase under the optimum production conditions we determined in our previous study, and we tried to determine some biochemical characteristics of the enzyme. Thus, this study is believed to contribute future research on the uses of the inulinase enzyme of *A. wentii*, which has not been known to synthesize inulinase in industrial applications.

Materials and Methods

Microorganism Used in the Study

A. wentii was used in this study was supplied by the Biology Department of Faculty of Sciences of Trakya University. The stock cultures obtained by growing in Potato Dextrose Agar (PDA) at 25°C for 5-7 days were stored at +4°C for use in the next stages of the experiment. The stock cultures were renewed by passage once a month.

Media used in the Study

The screening medium containing inulin as the only carbon source used by Derycke & Vandamme (1984) was redesigned to include Jerusalem artichoke powder instead of inulin and was used for inulinase production by *A. wentii* (Derycke & Vandamme 1984).

Inulinase Production and Recovery

The cultures in the stock were cultivated in sterile production media which was prepared in the form of 50ml in 250ml Erlenmeyer flasks (1% Jerusalem artichoke extract, 0.05% $MgSO_47H_2O$, 0.15% yeast extract, 0.1% KH_2PO_4, 0.023% NH_4NO_3). The culture media were left to grow in a water batch with a shaking speed of 100rpm at different temperatures and times according to the varying experimental conditions. The micella were filtered and collected from the growth medium after the fungal growth. The micella was dried in an 80°C oven and was measured in terms of dry weight. The crude filtrates were used as the enzyme source (Ertan et al. 2005).

Measurement of the Inulinase Activity

The inulinase activity was measured by incubating 0.1ml of the enzyme and 1ml of the buffer (0.1 M pH 5.0 sodium acetate buffer) substrate prepared in 1 ml of 0.1% inulin prepared in sodium acetate buffer at 35˚C for 10 minutes. The 3.5-dinitrosalisilic acid (DNS) method was used to identify the released reductor sugar. One unit of inulinase activity was defined as the amount of enzyme catalyzing the liberation of 1µmol fructose produced per min at pH 6.0 and 35˚C (Miller 1959).

Some Biochemical Parameters Affecting Enzyme Activity and a Preliminary Characterization of The Enzyme Extract

Incubation time, optimum temperature, thermostability, optimum pH and pH stability of the crude enzyme extracts were analyzed. Furthermore, an approximate Michaelis-Menten constant, K_m and V_{max} were determined for the enzyme extract. All tests were conducted in three replications.

Incubation pH, Temperature and Time

The enzyme substrate mixtures with pH values of 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0; acetate buffer for pH 3.0-5.0, phosphate buffer pH for 6.0-7.0; borate buffer pH for 8.0 were prepared to determine the optimum pH value of the crude inulinase of *A. wentii*. The inulinase activity in each sample was measured as U/ml and the optimum pH value was determined. Incubation was performed at 35°C for 10 minutes.

The enzyme substrate mixture was left to incubate in water baths at 25, 30, 35 and 40°C to analyze the effect of incubation temperature on activity. The incubation time was set to 10 minutes and the incubation pH was set to 6.0. The activity values were calculated in terms of U/ml after the incubation. The enzyme and substrate mixture were left to incubate at different times to determine the optimum incubation time. The time resulted in the maximum activity was selected as the appropriate incubation time (Ertan et al. 2005).

Calculation of the Km and Vmax Values of the A. wentii Inulinase

The enzyme activity was measured by using 0.1ml crude enzyme and substrate solution (0.1-0.7 mM inulin) prepared in increasing concentrations of 0.1 M pH 5.0 sodium acetate buffer. Reaction occurred at pH 6.0 and 35°C. The incubation time was set at 10 minutes. The K_m and V_{max} values of the *A. wentii* inulinase were calculated using a Lineweaver-Burk graph (Sanal et al. 2005).

The Inulinase Enzyme's pH and Thermal Stability

A 0.1 ml of buffer prepared at different pH values (3.0-8.0) was added to 0.1 ml of crude enzyme. The mixture was incubated at room temperature for 30 minutes. 1 ml

substrate solution with a pH value of 6.0 was added and the mixture was left to incubate at 35°C for 10 minutes. The enzyme activity was measured as U/ml at the end of the incubation time. The enzyme was incubated at different temperatures (30-80°C) for 20 minutes to determine the thermal stability of the crude enzyme. The activity was measured after incubation. The remaining activity was expressed as relative activity (Ertan et al. 2005, Sanal et al. 2005).

Statistical analysis

A Statview ANOVA program was used for statistical analysis of the data. The data was analyzed using two way analysis of variance at a significance level of $p<0.05$.

Results

The Effect of Incubation pH and Incubation Temperature on Inulinase Activity

The enzyme activity was measured at pH values varying from 3.0 to 8.0 in order to determine the pH value in which *A. wentii* crude inulinase enzyme worked the best. The pH value with the maximum inulinase activity (1.345U/ml) was found to be 6.0 (Figure 1).

The post-incubation activity values were measured at different temperatures from 25 to 40°C to determine the optimum incubation temperature. The incubation temperature with the highest inulinase activity (1.555U/ml) was found to be 35°C (Figure 2).

The Effect of Incubation Time on Inulinase Activity

The enzyme substrate mixture was left to incubate for different periods to determine the optimum incubation time for *A. wentii* inulinase. The enzyme activity was measured at the end of the reaction time. The highest enzyme activity (1.526 U/ml) was obtained at the end of 10 minutes of incubation.

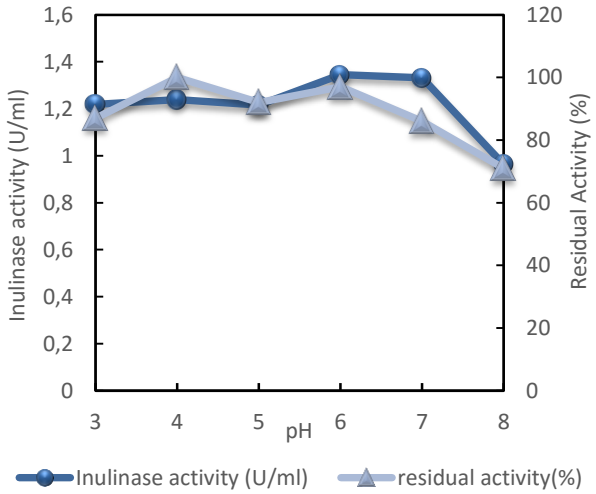

Fig. 1. Effects of incubation pH on inulinase activity and enzyme stability. Buffers with 0.1% (w/v) inulin; temperature: 35°C; incubation time: 10 min. The studied pH range was 3-8. The results are expressed in terms of the residual activity of the inulinase extract. Data represent the mean of three replicates.

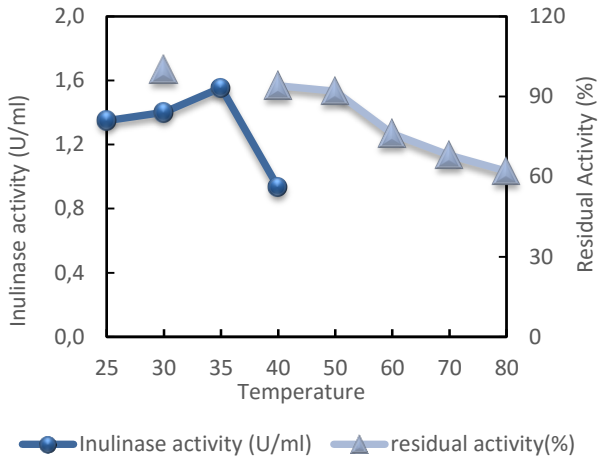

Fig. 2. Effects of incubation temperature on inulinase activity and enzyme extract stability. 35°C was the optimal temperature of the crude inulinase produced by *A. wentii*. Sodium acetate buffer (0.1 M, pH 5.0) with 0.1% (w/v) inulin; incubation time: 10 min. The studied temperature range was 30-80°C. The results are expressed in terms of the residual activity of the inulinase extract. Data represent the mean of three replicates.

The Effect of the Substrate Concentration on Inulinase Activity

The analysis of the appropriate substrate concentration for *A. wentii* inulinase showed that the inulinase activity increased until the substrate concentration reached 0.6 mM and remained stable at higher concentrations (Figure 3). The K_m value was calculated as approximately 1×10^{-4} M and the V_{max} value was calculated as 6.134 mmol/ml/min for *A. wentii* inulinase (Figure 4). The K_m value determined in this study is similar to the findings reported in the literature (Fullbrook, 1996).

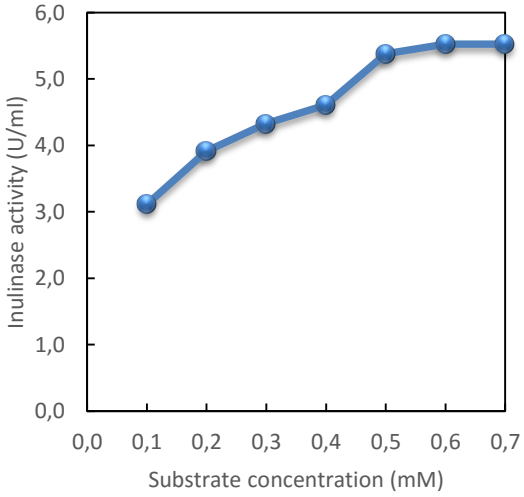

Fig. 3. The effect of substrate concentration on inulinase activity. Data represent the mean of three replicates.

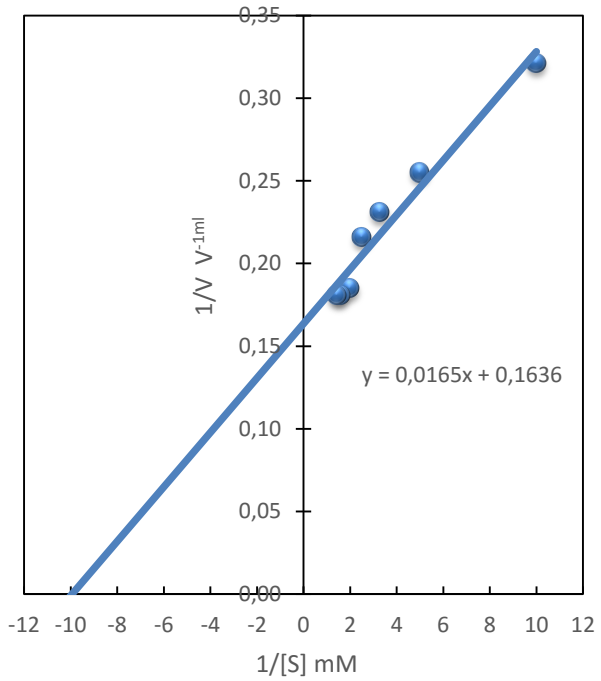

Fig. 4. Lineweaver-Burk graph. Data represent the mean of three replicates.

pH Stability and Thermal Stability

The relative activity of the inulinase enzyme at 3.0-6.0 pH intervals in a 0.1M sodium acetate buffer is presented in Figure 1. The crude enzyme extracts maintained their activity at a high level (98%) at pH intervals of 3.0-6.0. However, the activity was observed to decrease at pH 7-8. The thermal inactivation curve of the enzyme is presented in Figure 2. The inulinase enzyme maintained its activity at a high level until 50°C and began to decrease and had a relative activity of 62.3% at 80°C (Figure 2).

Discussion

The incubation pH was determined as 6.0 in experiments performed in order to determine the biochemical characteristics of the inulinase enzyme (Figure 1). Similarly, studies by different researchers with *Penicillium guilliermondii* reported that the optimum incubation pH of the inulinase enzyme obtained from this species was found to be 6.0 (Zhang et al. 2009). Mahmoud et al. (2011) reported the optimum pH within 6.5-7.5 for *Aspergillus niger* NRRL3, *A. amowori* MEA NRRL3112 and *A. oryzae* MEA NRRL 3487. The optimum pH for *A. versicolor* was reported as 5.5 and for *Penicillium janczewskii* within 4.8-5.0 (Sharma & Gill 2007). The exoinulinases obtained from bacteria are generally known to have higher incubation pH than yeasts and fungi, however, the optimum incubation pH of the exoinulinase obtained from *Geobacillus stearothermophilus* KP1289, a bacterial species, was found to be approximately 6.0 (Tsujimoto et al. 2003). In a study examined the appropriate incubation pH for the endoinulinase obtained from *Penicillum* sp. TN-88, maximum activity was observed at pH 5.2 (Nakamura et al. 1997). On the other hand, the optimum incubation pH was found to be 7.5 for the endoinulinase obtained from *Artrobacter* sp. S37 (Kang et al. 1998). This data shows that endoinulinases obtained from bacteria have higher incubation pH than endoinulinases obtained from fungi (Chi et al. 2009). However, the optimum incubation pH was reported as 5.5 for an endoinulinase obtained from *Pseudomonas* sp. (Kim et al. 2008). The optimum pH interval of the inulinase enzyme purified from yeasts and fungi was observed be in the range from 4.5 to 6.0 (Ge & Zhang 2005, Selvakumar & Pandey 1998, Singh & Gill 2006, Sheng et al. 2008). An analysis of the relationship between incubation temperature and inulinase activity revealed that maximum activity occurred at 35°C. Inulinases obtained from soil microorganisms generally show maximum activity below 50°C. The optimum temperature for inulinases generally varies in the range of 30-55°C (Chi et al. 2009). On the other hand, Zhang et al. (2009) and Sheng et al. (2008) observed an optimum incubation temperature of *P. guilliermondii* inulinase as 60°C (Zhang et al., 2009; Sheng et al., 2008). Endoinulinases obtained from yeasts and bacteria showed maximum activity generally at 50-55°C (Nakamura et al. 1997; Kang et al. 1998).

The V_{max}/K_m value is used in the literature as a measure of the catalytic activity of an enzyme and has been reported to be a useful parameter for choosing the most effective enzyme for industrial practices (Ortega et al. 2004). In this study, *A. wentii* inulinase activity was found to have a K_m and V_{max} values of 1×10^{-4} M and 6.09U/mmol/ml/min, respectively, at optimum conditions and in different substrate concentrations. Arand et al. (2002) found *Aspergillus awamori*'s K_m value as 0.003mM. Mutanda et al. (2009) found K_m and V_{max} values as 4.75mM and 833.3 µmol $min^{-1}mg^{-1}$, respectively in a study on *A. ficuum*. Our values are consistent with the K_m intervals (0.01-100mM) determined for the inulinases of other microorganisms with industrial uses reported in the literature (Fullbrook 1996).

The enzyme was left to incubate for 30 minutes with different pH values to determine the pH stability of the inulinase enzyme. The activity loss was found to be lower at the pH interval of 4.0-6.0 at which the enzyme maintained 92.2% of its actvity, but the activity decreased above pH 7 (remaining activity was 85.1%). The inulinase enzyme obtained from *P.* was found to maintain 95.1% of its activity within pH 4.0-9.0 range for a period of 2 hours, while it showed a significant activity loss at pH values higher than 7.0 (Zhang et al. 2009). Treichel et al. (2009) reported that *Kluyveromyces marxianus* NRRL Y-7571 inulinase enzyme maintained its activity at 70% with a pH of 4.4 after 82 hours, while there was 35.2% activity loss at pH 4.0. An examination of pH stability during enzymatic reactions of microbial inulinases revealed that activity was generally maintained at pH 3.5-6.5. Zittan (1981) and Cazetta et al. (2005) found the optimum pH as 4.0 for *K. marxianus* and reported that activity loss increased at higher pH values and no enzyme activity was observed at pH 10.5.

An inulinase activity measurement after 20 minutes of incubation at different temperatures (30-80°C) to analyze the effect of high temperatures on inulinase activity revealed that *A. wentii* inulinase maintained 62.3% of its activity at 80°C. This is an important finding in terms of industrial use. Lima et al. (2009) observed that *K. marxianus* CCMB 322 inulinase lost its all enzyme activity at 80°C. In studies in which purified enzymes were used, there was no significant loss in activity of *Streptomyces* sp. inulinase at 70°C after a 6-hour incubation and 75% of its activity was maintained after a 12-hour incubation. Inulinase activity was reported to decrease rapidly at 80°C (50%) (Sharma & Gill 2007). The inulinase enzyme obtained from *Scytalidium acidophilum* was reported to be a thermo-stabile enzyme and to maintain 95% of its enzyme activity at 60°C after 6 hours carried out a study with *A. niger* and reported that after a 30-minute incubation at different temperatures (20-80°C) activity was completely maintained at 60°C and was at approximately 60% at 80 °C (Kim et al., 1994; Kumar et al., 2011). Chen et al. (2009) incubated an inulinase enzyme obtained from *A. ficuum* at different temperatures (40-90°C) for one hour (Chen et al., 2009) The researchers observed that a 80% activity was maintained at 50°C and a 60% decrease occurred in activity at 60°C. The activity was totally lost after one hour of incubation at 80°C (Chen et al., 2009). The inulinase of the marine fungus *C. aureus* G7 was quite stable at 65°C (Cazetta et al., 2005).

In conclusion, inulinases are enzymes that can be used for industrial purposes. Reaction products obtained from the use of inulinase can be used for various purposes in food industry. In addition, the substances that are used as substrates are a significant way to make use of waste materials. In our study, we determined some biochemical characteristics of *A. wentii* inulinase that have not been previously analyzed. Our research and experiment results constitute a first step toward enzyme purification studies. We believe that *A. wentii* inulinase can be an enzyme with industrial uses due to its relationship with its substrate and its good pH stability and thermal stability. *A. wentii* inulinase can also be a new and usable source for preparation of pure fructose from inulin-containing agricultural products.

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