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Research Article

Investigation on the activity of purified inulinase enzymes from fungal strains on the inulin extracted from chicory (*Cichorium intybus*) for production of fructose sweetener

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ABSTRACT

Chicory is a rich plant source for the prebiotic inulin. The aim of this study was to evaluate the activity of purified inulinase enzyme from fungal strains on the inulin extracted from chicory. Inulin was extracted by soaking in water and precipitating in ethanol. Fungi were isolated from the soil around chicory root and screened for inulinase production along with fungal strains, *Kluyveromyces marxianus* PTCC 5006 and *Aspergillus flavus* PTCC 5304. The high inulinase-producing fungal isolate was identified based on the sequencing of 18S rRNA gene. The enzyme was purified using ammonium sulfate-polyacrylamide and dialysis. The molecular weight of the protein was detected using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). One fungal isolate that showed high enzymatic activity (2233 U/ml) was obtained. This isolate was identified as the species *Talaromyces moniliformis*. Among the collection strains, *Kluyveromyces marxianus* had the highest inulinase production; while its inulinase activity was lower than that of *Talaromyces moniliformis*. The results from enzyme purification from *Talaromyces moniliformis* showed that the most specific enzyme activity (546.6 U/mg) and the highest purification yield (62.17%) were obtained by the precipitation in 55% ammonium sulfate. Dialysis of the protein resulted in the extraction of a protein with a reduced specific enzyme activity (188.6 U/mg). The molecular weight of the purified protein was estimated at15 kDa.The fungal inulinase which purified from native resources in the present study can be used in food and pharmaceutical industries due to its high enzymatic activity.

1. Introduction

Today, new sources for low-calorie sweeteners have been requested. Most nutritionists agree that sugars may not always be replaced by many other food elements in terms of nutritional value (Pourfarzad et al., 2015; Carlson et al., 2018).

Inulin is a poly-fructan composed of linear poly fructose chains with 1,2-βbonds. Inulin is stored carbohydrate in a variety of plants and is found in more than 30,000 plant products. Its rich sources include the roots and tubers of plants such as artichokes, chicory and dahlias (Wichienchot et al., 2010). Each plant-originated inulin chain consists of 2 to 100 fructose units and has been considered as one of the best sources for the production of high fructose

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syrup. This syrup contains a D-fructose content of 15% more than that of the syrup yielded by a multi-enzymatic system for starch hydrolysis. High fructose syrup is usually produced by the isomerization of corn starch hydrolyzates in a two-step process. This conversion requires three different enzymes: alpha-amylase, glucoamylase, and glucose isomerase. In the first stage of enzymatic isomerization, hydrolysis of starch occurs, during which a mixture containing 42% fructose and 50% glucose is obtained. During the second stage, the mixture content is reached 95% fructose. An alternative method is to produce high fructose syrup by enzymatic hydrolysis of fructan polysaccharides such as inulin in a single step (Guo et al., 2014). Inulinase is one of the most important industrial enzymes that is produced by a large number of organisms including molds, yeast, bacteria, and some plants (Das et al., 2019). Industrial production of the enzyme inulinase by fungi is mostly done by *Aspergillus* and *Clauromycus* species on inulin as the most important substrate (AbdAl-Aziz et al., 2012). Also, it has recently been found that *Penicillium* species, produce inulinase in addition to the high ability to produce other hydrolytic enzymes such as xylanase (Terroneet al., 2018; Corradoet al., 2021).

Chicory is one of the most important sources for inulin production due to its high ratio of inulin to dry content (Preedyet al., 2013). Chicory is native to the Mediterranean, Central Asia, and North Africa although is grown in many countries including the United Kingdom, Belgium, France, Netherlands, Germany, South Africa, and India (Darjaniet al., 2016). The present study aimed to propose an Iranian native plant source (chicory) for inulin production and also use native fungal species to produce inulinase enzyme, which then was purified from the protein content of the production medium.

2. Materials and Methods

2.1. Extraction of inulin from chicory root

Whole chicory roots along with the soil around the roots were collected from farms in western Isfahan, Iran, and transferred to the research laboratory in Falavarjan Branch, Islamic Azad University, Isfahan, Iran. The roots were peeled and washed, and then was dried in a tray for several days. The dried roots then were crushed and ground into a powder by a mill. After that, 5 g of a root plus 45 ml of distilled water was exposed to an 80 °C heat for one h. After filtration of the obtained extract, its pH was raised from 0.5-6 to about 10-12 using calcium hydroxide solution. Then it was placed at 50 °C for 30 min and the precipitate was separated by a Whatman No 1 filter paper. The pH of the extract was raised to 8-9 with 10% phosphoric acid solution and then the extract was placed at 60 °C for 2 h to be precipitated. After that, 0.12 g activated charcoal was added to it and after stirring vigorously, it was kept at 60 °C for 15-30 min. Then the activated charcoal was separated by a Whatman No 1 filter paper. The extract was then concentrated up to the brix 42 using a vacuum condenser. After that,16 mlof ethanol 96% was added to the concentrated extract and for complete sedimentation, the mixture was exposed to a 40 °C heat for two days. In the next step, the ethanol was evaporated by placing the precipitate at 50 °C for 4 days. In the end, the dried sediment was ground and its final weight was measured (Mohsenzadehet al., 2016). All chemicals were purchased from Merck Company (Germany).

2.2. Inulinase producing fungi

The soil around the chicory root was separated and poured into sterile glass containers. Potato dextrose agar (PDA) medium (HiMedia, India) was used to isolate the fungi. For this purpose, serial dilutions were prepared from soil samples and then 0.5 ml of each dilution was poured into the center of the PDA medium and streaked in several directions with a sterile glass spreader. The culture media were then incubated at 30 °C for one week. After that, the morphology of the grown colonies was investigated and the colonies were purified on PDA media. Then the microscopic characteristics of the purified colonies were analyzed following the slide culture method by staining with lactophenol cotton blue (Merck, Germany) (Rosana et al., 2014). Also, the collection of fungal strains including *Clevermycin marxians* (PTCC 5006) and *Aspergillus flavus* (PTCC5304) were prepared as lyophilized ampoules from the Persian type culture collection, Iran.

2.3. Screening of inulin degrading fungi on inulin-enriched medium

The selective inulin agar medium containing 1.5% inulin (as the sole carbon source), 1% peptone, 1% $(NH_4)_3PO_4$, 0.5% NaCl, 0.1% KH_2PO_4, 0.05% $MgSO_4$, and 1.5% agar (pH = 6-7) was prepared. Inulin suspension was separately prepared in 15 ml distilled water and sterilized in an autoclave at the pressure of 10 Ib/In^2. The rest of the medium components were sterilized in an autoclave at the pressure of 15 Ib/In^2 and then was added to the inulin suspension. Each sample dilution was cultured as a spot on the agar platesand incubated at 30 °C for 4 days. After that iodine solution was poured on the surface of each plate and kept on a horizontal level for 3 to 5 min. Then the plates were washed 2-3 times with distilled water and kept open for 10-15 min. Finally, the inulinase-producing isolates were detected by investigation of clear zone formation around the colonies, because of inulin degradation by the fungi (Mansouri et al., 2013; Abou-Talebet al., 2019). All chemicals were purchased from Merck Company (Germany).

2.4. Enzyme activity assay

The amount of reduced sugar released during the enzymatic reaction was determined by end-point spectrophotometry using 3,5dinitrosalicylic acid which detects the presence of free carbonyl groups (C = O) related to reducing sugars. The reaction was performed in the presence of 1% inulin substrate at a temperature of 39-40 °C for 60 min in sodium acetate buffer with a favorable pH = 5.5-6. Enzyme activity was stated as µmol of reducing sugar produced in the reaction mixture each min. Specific enzyme activity was calculated as the number of enzyme units per ml divided by the concentration of protein in mg/ml (U/mg) (Miller, 1959; Mansouri et al., 2013; Emtiazi et al., 2001). All chemicals were purchased from Merck Company (Germany).

2.5. Protein assay

To 100 µl of the solution, 5 ml of Bradford reagent was added at room temperature, and then the optical density (OD) of the content was detected after 5 min at the wavelength of 595 nm. The amount of protein (mg/ml) was measured according to the standard curve of bovine serum albumin (sigma-aldrich, USA) (Bradford, 1976).

2.6. Molecular identification of isolates

Universal primers including ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used to amplify the 18SrRNA gene. The PCR mixture with a final volume of 25 µl was included PCR buffer (1X), dNTPs (1.5 mM), $MgCl_2$ (0.2 mM), forward and reverse primers (5 Pmol/µl each), and Taq DNA polymerase enzyme (1.25 U) (SinaClon, Iran). PCR reaction was includedan initial denaturation step (96 °C, 5 min), followed by 35 cycles including denaturation step (94 °C, 30 s), primer annealing (50 °C, 30 s) and elongation (72 °C, 1 min), and a final elongation step (72 °C, 10 min) (Mirhendi et al., 2010). The PCR product size was evaluated on 1% agarose gel electrophoresis. After examining the quality of the PCR product resulting from the amplification of the desired fragment, the PCR product was sent to Royanzistgene Company (Iran) for sequencing. Sequencing results were checked by Chromas software and then evaluated using BLAST server in NCBI database and the species that attributed to the isolate was identified.

2.7. Enzyme purification

The selected fungus was inoculated to a selective inulin broth medium with the same ingredients (except agar), and the same preparation method as selective inulin agar medium. The inoculated medium was incubated at 30 °C and 120 rpm for 3 days. Afterward, the culture medium was centrifuged at 4 °C for 15 min at 12000 rpm. Then the precipitate was discarded and 40% (w/v) ammonium sulfate was added to the supernatant and dissolved on a magnetic stirrer at 4 °C. The solution was kept at 4 °C for 24 h and the obtained precipitate was separated from the supernatant by centrifugation at 4 °C for 15 min at 12000 rpm. Finally, the enzyme activity and protein content of both supernatant and pellet was separately assessed. In the next step, 55% ammonium sulfate was added to the supernatant and the

previous steps were repeated. The steps were also repeated with 70% ammonium sulfate saturation. Finally, the collected precipitate was dialyzed using dialysis tubing cut off 12000 to 14000 Daltons (684212, Carolina Biological Supply Company) in phosphate buffer 0.5 M. To prepare dialysis bags, the bags were first boiled in a 5% solution of sodium carbonate for 15 min. Then the bags were washed twice in distilled water. After that, the drained bags were boiled in 0.05 M EDTA solution for 15 min and rinsed again with distilled water. After preparation of the bags, the precipitates were dissolved in phosphate buffer and poured into them and the bag was placed in phosphate buffer. The buffer was changed several times during 24 h. Finally, the enzyme activity and protein content of dialyzed precipitate was assessed. The purification yield in each stage was calculated by dividing the specific enzyme activity of the purified protein by the specific enzyme activity in crude medium extract× 100 (Kumar et al., 2012; Tripathi et al., 2014). All chemicals were purchased from Merck Company (Germany).

2.8. Evaluation of purified protein by SDS-PAGE

The method was used to detect the relative molecular weight of the protein. For this purpose, first, the samples were stirred for 30 min at the temperature of 2 °C and after placing them in a water bath at 95 °C for 3 min, they were centrifuged at 1000 rpm for 1 min. Then the supernatant was separated and stored at -20 °C until electrophoresis. For SDS-PAGE assay, 750 µl of extraction buffer containing 0.625 M tris-hydrochloric acid, 10% sodium dodecyl sulfate, 5.2% β-mercapto ethanol, 7% glycerol, and 4 mg bromophenol blue were added to the samples, and electrophoresis was performed by an SDS-PAGE apparatus (Bio-Rad TGX) with the voltage of 200 V (Maizel, 2000). All chemicals were purchased from Merck Company (Germany).

3. Results

3.1. Inulin extracted from chicory root

The extracted inulin was a white powder (Fig.1) with a final weight of 0.64 g. The efficiency of the yielded dry matter was 90%.

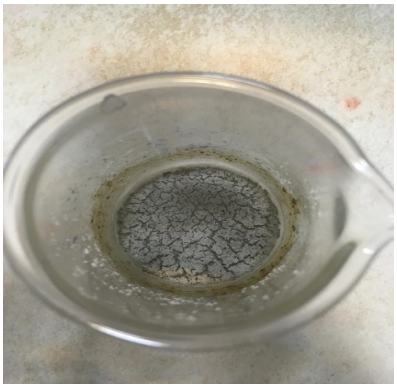

Figure 1. The inulin which was extracted from chicory root

3.2. Screening of inulinase producing fungi

Inoculation of soil samples into PDA medium resulted in the isolation of 4 fungal isolates. Screening of the isolates in the selective agar medium containing inulin resulted in the creation of a significant clear zone (15 mm) around the colonies of one isolate (Fig.2). A macroscopic view of the fungal isolate revealed green colonies with white borders and powdery mildew, and the microscopic analysis of the isolate detected transparent hyphae including septa creating simple or branched conidiophores. The results indicated that the fungus may belong to the genus Penicillium. Among the studied collection strains, Kluyveromyces marxianus created a greater degradation zone (10 mm) than that of Aspergillus flavus (8 mm) in the screening medium.

3.3. Molecular identification of inulinase producing fungal isolate

Amplification of the 18SrRNA gene sequence using the universal ITS1 and ITS4 primers revealed a 400 bp band in agarose gel electrophoresis (Fig.3). The evolutionary distance tree was plotted based on the nucleotide substitution with the maximum difference of 0.75 from the neighbor binding method. The compliance of the 8 index sequences is shown in Fig.4. As the results show, the isolate was related to Talaromyce smoniliformis from ascomycetes.

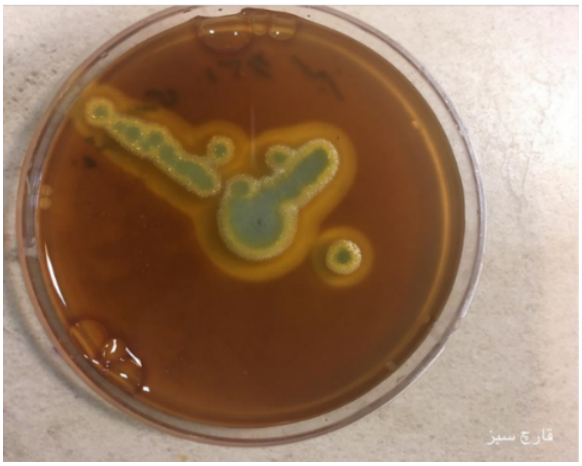

Figure 2. Results related to the growth of the selected fungal isolate in the inulin agar medium. The presence of a clear zone around the colonies after the addition of iodine indicates the production of inulinase.

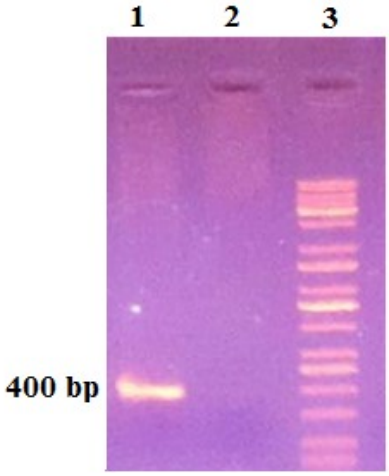

Figure 3. Results from agarose gel electrophoresis of the amplified 18S rRNA gene sequence in the inulinase-producing isolate revealed a 400 bp band (well 1) compared to the negative control (well 2) and a 100 bp DNA marker (well 3).

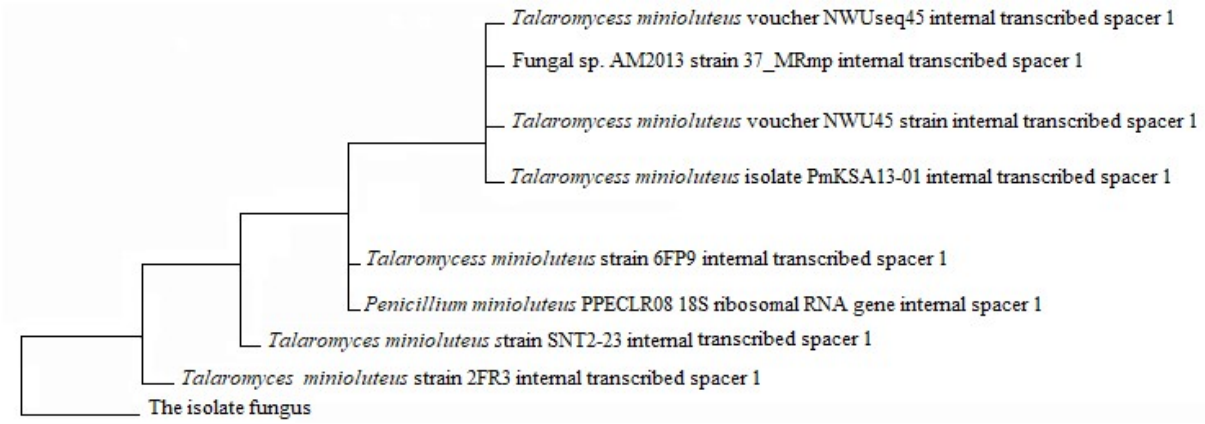

Figure 4. The phylogenetic tree result for identification of the inulinase-producing isolate (*Talaromyces moniliformis*)

3.4. Comparison of inulinase activity between the 3 fungal strains

The results from enzymatic activity assays for the three inulinase-producing fungi including *Talaromyces moniliformis*, Kluyveromyces marxianus, and Aspergillus flavus at 24, 48, 72, and 96 h incubation times in inulinase production medium are shown in Fig. 5. These results showed that after 24 h incubation, the highest enzymatic activity were related to *Talaromyces moniliformis*, the fungus which was isolated in the present study. The highest enzymatic activities after 48, 72, and 96 h incubation times was related to Kluyveromyces marxianus with no significant difference with the enzyme production by *Talaromyces moniliformis*. All three fungi reached to their highest enzymatic activities at 72 h incubation time, although showed a significant decrease after 96 h incubation in inulinase production medium.

3.5. Results from inulinase purification steps

The results obtained from enzyme purification following precipitation in different concentrations of ammonium sulfate and dialysis step are shown in Table 1.

The highest enzymatic specific activity (546.6 U/mg) was obtained by55% ammonium sulfate saturation, and the highest purification yield (62.17%) was obtained in the same stage. In the dialysis stage which was performed on the precipitate obtained by55% ammonium sulfate saturation, the enzyme activity was determined to be 188.6 Units per mg/ml protein. These results showed that precipitation with ammonium sulfate has led to the concentration of protein and to the increased enzymatic activity; although a large part of enzymatic activity (about 78.55%) has been lost by the dialysis of the precipitate. Therefore, precipitation with 55% ammonium sulfate saturation was the best method for partial purification of the enzyme.

3.6. Results from SDS-PAGE gel electrophoresis of the purified inulinase enzyme from different purification stages

The results showed that the purified protein had a molecular weight of about 15 kDa. Purification of the protein by ammonium sulfate saturation resulted in the sharpest band indicating the highest amount of purified protein obtained in this stage (Fig. 6).

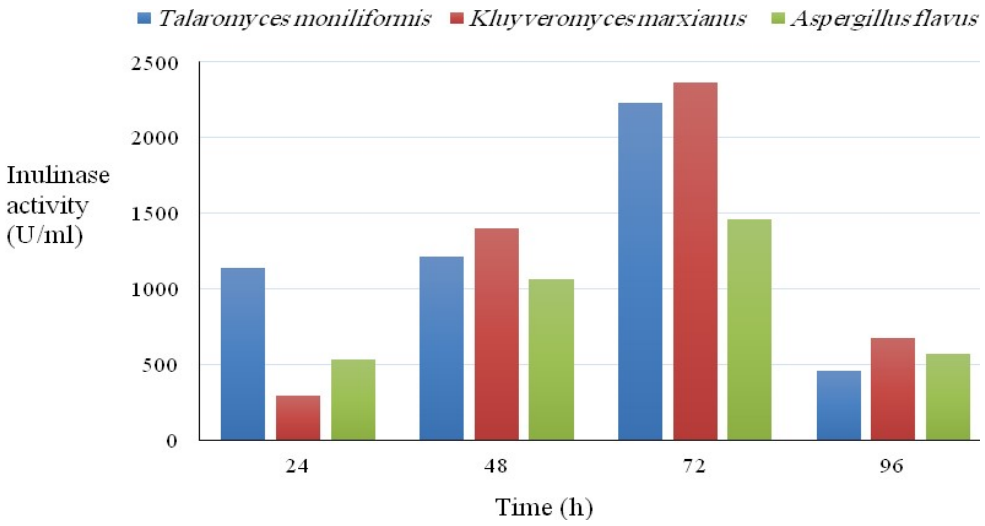

Figure 5. Results related to the measurement of inulinase activity in three fungal strains (*Talaromyces moniliformis*, Kluyveromyces marxianus, and Aspergillus flavus) at different incubation times in inulinase production medium.

Table 1. Results from purification steps performed on the inulinase obtained from *Talaromais moniliformis*. Purification yields are calculated in the protein precipitates and the dialyzed protein.

Step	Volume (ml)	Protein content (mg/ml)	Inulinase activity (U/ml)	Specific enzyme activity (U/mg)	Purification yield (%)
Crude medium extract	100	2.54	2233	879.1	-
Supernatant (40% ammonium sulfate saturation)	98	3.847	1776	461.6	-
Precipitate (40% ammonium sulfate saturation)	1.5	34.3	18288	533.18	60.64
Supernatant (55% ammonium sulfate saturation)	96	47.37	15210	321.1	-
Precipitate (55% ammonium sulfate saturation)	1.5	33.7	18419	546.6	62.17
Supernatant (70% ammonium sulfate saturation)	94	33.74	15217	451.1	-
Precipitate (70% ammonium sulfate saturation)	1.5	32.513	15827	486.78	55.36
Dialyzed protein	20	1.29	242.3	188.6	21.45

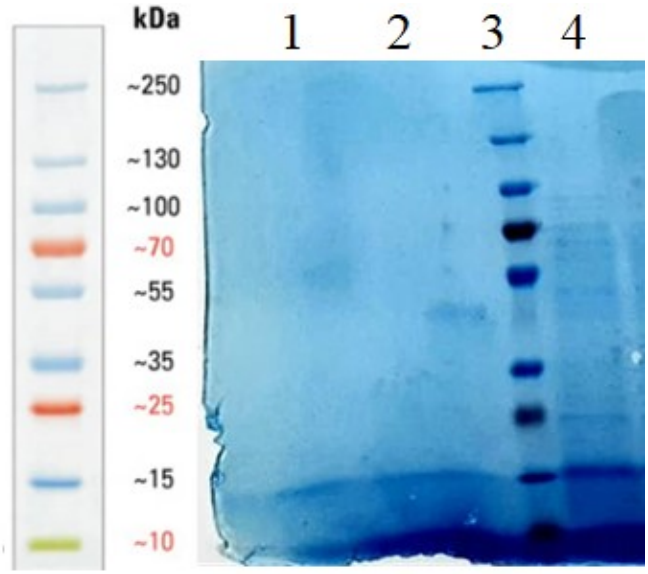

Figure 6. Results from SDS-PAGE of inulinase enzyme in different purification stages. 1: crude sample, 2: dialyzed protein precipitate, 3: 10 kDa marker, 4: the protein precipitate by 55% ammonium sulfate saturation.

4. Discussion

In the present study, inulin was extracted from native wild chicory. The extracted inulin was a white powder with a final weight of 64.0 g and dry matter content of 90%. In the study of Terkmane et al. (2016), the efficiency of inulin extraction from fresh chicory root after purification was estimated to be about 21.10%. In the study of Li et al. (2018), 23.13% of inulin was extracted from dried artichoke powder under optimal water extraction conditions. The inulin yield of chicory root in this study was higher than the two above reports. In a study, concentrations of 40%, 60%, and 80% ethanol were used to extract inulin from chicory root and it was observed that the extraction with 80% ethanol caused the highest amounts of inulin obtained. Also, the amount of fructan extracted in the presence of 40% and 60% ethanol was the highest, which indicates that increasing the concentration of ethanol would be effective in increasing fructan extraction (Li et al., 2012, Zimmermann and Kaltschmitt, 2022). In the present study, the concentration of 96% ethanol was used to extract inulin and a high amount of inulin was extracted. According to these results, the method of inulin extraction by a high percentage of ethanol can be considered as one of the most suitable extraction methods.

Based on the results obtained in this study, the strain of *Talaromyces moniliformis* isolated from the soil around chicory root, formed a clear zone on inulin agar indicating inulinase enzyme production. Collection strains of fungi including *Kluyveromyces marxianus* PTCC 5006 and *Aspergillus flavus* PTCC 5304 NO also were selected for analyzing inulinase production based on the previous reports for inulin degradation (Vinoth Kumar et al., 2011; Fawzi, 2011; Galindo-Leva et al., 2016; Singh et al., 2016; Das et al., 2019). Only *Kluyveromyces marxianus* showed a clear zone of degradation on inulin agar. Inulinase activity by the 3 fungi at 24, 48, 72, and 96 h growth in inulin broth showed that after 72 h, the enzyme activity decreased by all three fungi. Galindo-Leva et al. (2016) showed that Kluyveromyces marxianus isolated from chicory grew well and expressed the ability to produce inulinase in the inulin-rich medium. Measurement of the activity of this enzyme showed that the highest level of activity was in 72 h) which is coincident with the results obtained in the present study. In a study on isolating and determining inulinase producing strains from chicory, among 15 fungal isolates, only 3 strains showed the ability to produce inulinase on an inulin-rich medium. By using enzyme production assessment by thin-layer chromatography, 3 fungal strains with high inulinase activity were identified in their study. Micro-morphological and macro-morphological characterization, as well as molecular identification of BGPUP6 isolate, which showed the maximum enzymatic activity, detected that the fungus was related to *Aspergillus tritici* (Singh et al., 2016). Golunski et al. (2014) investigated the isolation of inulinase producing fungi from the sheng plant. Out of 25 fungal isolates which were grown on a PDA medium, only 10 isolates showed the ability to produce inulinase on an inulin-rich medium. Purification and measurement of enzyme activity were performed using gel filtration chromatography. Molecular identification showed that most isolates belonged to the genus Penicillium, and Aspergillus. In the present study, *Talaromyces moniliformis* strain isolated from chicory showed the ability to produce an inulinase enzyme. This genus has previously been in the genus Penicillium (Yilmaz et al., 2014).

In general, in different studies, fungal strains have been more efficient in producing inulinase enzyme than bacterial and mold strains, but few studies have been done on them (Surtiand Mhatre, 2021; Sunarti et al., 2022). Therefore, further studies are needed to find different inulinase-producing fungal isolates and to optimize their activities as well as the purification of the produced inulinase enzyme by different methods.

The results from the purification of inulinase obtained from the most productive fungus, *Talaromyces moniliformis* showed that the maximum enzyme yield (62.17%) was obtained in the precipitation stage using 55% ammonium sulfate saturation and the lowest enzyme yield (21.45%) was seen in the dialysis stage. Vinoth Kumar et al. (2011) has also shown that ammonium sulfate saturation was the best purification method for the purification of inulinase produced by Aspergillus niger. They used 30% ammonium sulfate saturation and recovered 88% of the enzyme activity. The

enzyme was purified from culture filtrate to about 67-fold purity by $(NH_4)_2SO_4$ precipitation followed by four consecutive column chromatography steps. The purified enzyme is a single peptide with an approximate molecular mass of 73 kDa as analyzed by gel filtration and 70.8 kDa as assessed by SDS-PAGE. Ammonium sulfate did not block the activity of enzymes in both studies. Fawzi et al. (2011) obtained a decreased inulinase activity following ammonium sulfate saturation and used gel filtration and ion-exchange chromatography for further recovery of purified enzyme. Other technics also have been used for the purification of microbial-produced inulinases. Temkovs et al. (2018) used an aqueous two-phase system (ATPS) by concentration with polyethylene glycol (PEG) and mineral salts. An ATPS containing 26% PEG1000 and 26% $MgSO_4$ showed the most purification yield (66.17%) (Temkov et al., 2018). Zhang et al. (2020) purified the recombinant exo-inulinase InuAGN25 by using immobilized metal affinity chromatography and accessed to higher enzyme activity in low temperature by a mutation in the coding gene. In the present study, the enzymatic protein was largely lost in dialysis. The close sizes of dialyzing membrane pores and the size of protein (about 15 kDa) might be the reason for falling down the enzyme activity. Other studies have been detected fungal inulinase enzymes with different molecular weights by the SDS-PAGE method, from low molecular weight similar to this study up to 78 kDa in previous studies (Fawzi, 2011; Kholiavka et al., 2014).

Conclusion

The results of the present study showed that the fungus which was isolated from chicory root has a high ability to produce inulinase enzyme. This fungus which belonged to the species *Thalaromyces moniliformis* is reported for the first time as a novel inulinase producing fungus native to Iran. Also, by the cultivation of chicory through progressive methods such as tissue culture as well as extracting inulin from the plant by using the proposed method in the present study, this compound can be used for the industrial production of inulinase enzyme on an industrial scale.

Conflict of interest

None

Refereces

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