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High-cell density cultivation of recombinant *Pichia pastoris* harboring fungal endoinulinase and prebiotics applications of inulo-oligosaccharides

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Authors Info

**M. Surendarbabu and
S. Meenakshisundaram***

Centre for Biotechnology,
Anna University,
Chennai-600 025, India

*Corresponding Author Email :
meenakshi@annauniv.edu

Edited by

Dr. Sumati Gaumat

Reviewed by

Dr. G. Baskar
Dr. R. B. Raizada

Abstract

Aim : The objective of the present study was to clone and express *A. niger* endoinulinase gene in *P. pastoris* for high-level expression. Further to explore high cell density cultivation, biochemical characterization of recombinant endoinulinase and application of inulo-oligosaccharides (IOS) as prebiotics was also studied.

Methodology : Molecular cloning of *A. niger* endoinulinase gene in *P. pastoris*, screening of positive clones by genomic DNA PCR, shake flask studies, high cell density fermentation performed with both conventional and temperature shift approach, biochemical characterization of endoinulinase and *in-vitro* fermentation of IOS was carried out to confirm prebiotic efficacy.

Results : The endoinulinase gene of 1482 bp from *Aspergillus niger* was genetically engineered in the GS115 host and was secreted extracellularly using α signal sequence. As a result of fermentation with the conventional approach, recombinant endoinulinase activity was enhanced to 65.7 U ml⁻¹. Recombinant endoinulinase showed absolute substrate specificity for inulin, hydrolyzing inulin to IOS with the DP range 3-4.

Interpretation : Hydrolysis of inulin by recombinant endoinulinase was characterized. *In-vitro* fermentation of IOS by lactic acid and bifidogenic bacteria was studied as a part of industrial application and functional properties of IOS was similar to commercial prebiotics.

Key words: *Aspergillus niger*, Endoinulinase, Inulin, *Pichia pastoris*, Prebiotics

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Introduction

Endoinulinase is a commercially important enzyme used for the fructo-oligosaccharides production. Endoinulinases hydrolyses inulin by endocleavage, producing inulo-oligosaccharides, whereas exoinulinase enzyme hydrolyses inulin by exocleavage to produces fructose as a major product. Prebiotics like fructo-oligosaccharides has an extensive range of applications ranging from aquaculture to food industries. The major problem in aquaculture nowadays is infectious diseases in fish as they create enormous loss. Prebiotic triggers innate immune response in fish, for example, it increases fish growth and also activates complement system (Carbone and Faggio, 2016). Fructo-oligosaccharides have greater functional properties as prebiotics, dietary fiber and Bifidus stimulating factor (Skowronek and Fiedurek, 2004). Oligosaccharides nowadays are broadly used as functional foods (Ladirat *et al.*, 2014). Due to various application of oligosaccharides, demand for isomalto-oligosaccharides (Ojha *et al.*, 2015), cello-oligosaccharides (Chu *et al.*, 2014), xylo-oligosaccharides (Moura *et al.*, 2007), galacto-oligosaccharides (Ladirat *et al.*, 2014), and fructo-oligosaccharides (FOS) (Gern *et al.*, 2001; Ghazi *et al.*, 2006) have improved rapidly. FOS is synthesized from inulin and sucrose by enzymatic hydrolysis (Nakamura *et al.*, 1997) particularly, inulin derived FOS is mentioned as inulo-oligosaccharides (IOS). Lower Eukaryote yeast like *P. pastoris* is a significant host system for the expression of genes from heterologous sources (Cereghino and Cregg, 2000). Recombinant lipase expressed in different strains like *Escherichia coli*, *S. cerevisiae* and *P. pastoris* were compared to evaluate a better recombinant expression system. *P. pastoris* was evaluated as a potential host system for recombinant lipase production because of its better expression efficiency when compared with others recombinant strains (Valero, 2012).

Pichia pastoris has various advantages like it can easily grow in basal salt media, reach a very high cell density, and build up high concentrations of extracellular protein (Huang *et al.*, 2003). In addition, *P. pastoris*, as an extensive expression system, can perform protein processing, folding, and post translational modifications. As endoinulinase has gained massive interest in both health and food industry, various microorganisms are used for the feasible production of endoinulinases such as *Penicillium* spp. (Nakamura *et al.*, 1997), *Arthrobacter* spp. (Montesino *et al.*, 1998), *Aspergillus ficuum* (Chen *et al.*, 2012) and *A. niger* (Gern *et al.*, 2001). *A. niger* (DSM 2466) is one of the best producer for endoinulinase from isolated fungal strains which produces endoinulinase (Gern *et al.*, 2001).

In this study, *A. niger* endoinulinase gene was cloned and expressed extracellularly in *P. pastoris* GS115. Fermentation was performed with both conventional and temperature shift approach to improve the recombinant enzyme expression level. *In vitro* fermentation of prebiotic inulo-oligosaccharides by lactic acid and bifidogenic bacterial strains was also studied to check the prebiotic efficacy of IOS produced.

Materials and Methods

Strains and culture media : *A. niger*/DSM 2466 was procured from German collection of microorganisms and cell cultures (DSMZ). *E. coli* DH5 α (maintenance host), *Pichia pastoris* GS115 strain (expression host) and *pPICZ α B* (expression vector) were purchased from Invitrogen Co. Ltd. Restriction enzymes, DNA polymerases and T4 DNA ligase were purchased from Biolabs (New England Biolabs, USA). Fructo-oligosaccharide and Inulin were purchased from Sigma-Aldrich Co. Ltd. Plasmid extraction, PCR purification and gel elution kits were procured from Qiagen. All Medias used in this study were prepared according to the Invitrogen- *Pichia* Process Guidelines.

Construction of recombinant Plasmid : To obtain mature endoinulinase, 23 amino acids of the signal peptide (MLNPKVAYMVWMTCLGLTLPSQA) were removed. DNA sequence encoding the endoinulinase gene mature region without the predicted signal sequence was amplified by PCR using suitable sets of primers INU1 forward - CGGAATTCAGTCTAATGATTACCGTCC and INU1 reverse- GAATGCGGCCGCTCATTCAAGTGAAACAC. After total RNA isolation from *A. niger*, cDNA was synthesized. The synthesized cDNA was used as a template for PCR reactions. Recombinant plasmids *pPICZB-INU1* was constructed using PCR amplified INU1 gene from *A. niger*.

Transformation of constructed vector in *Pichia pastoris* : The linearized *pPICZ α B-INU1* plasmid construct of 10 μ g was mixed with 80 μ l competent cells in electroporation cuvette (0.2 cm) and pulsed (1500 V, 250 Ω , 50 μ F) using ECM@630 electroporator (BTX, San Diego, CA, USA) as per Invitrogen manual. Post electroporation, the positive transformants were screened using Zeocin antibiotic resistance in Yeast Peptone Dextrose agar medium. To further confirm the positive transformants, genomic DNA of resistant colonies was isolated and checked for the presence of plasmid construct by PCR using gene-specific primers.

Expression analysis in shake flask cultivations : Expression study of recombinant *P. pastoris* strains was performed in shake flask. Once the cells reached mid-log phase, cells were harvested from a 50 ml flask, centrifuged and transferred to 15 ml expression medium (Basal methanol medium). Induction was performed with methanol concentration of 1% (v/v) for 120 hrs at an interval of 24 hrs. The recombinant endoinulinase expression was analyzed by endoinulinase enzyme assay (Chen *et al.*, 2009) and the amount of protein expressed after induction was detected by SDS PAGE.

Bioreactor cultivation : Fermentation studies were carried out using a Basal Salt Medium supplemented with glycerol as major carbon source during growth phase and induction of AOX gene which is present upstream to gene sequence was triggered by methanol for the production of endoinulinase enzyme. A 3.7 l bioreactor (KLF2000, Bioengineering AG, Switzerland) with initial working volume of 2 l was equipped with online pH and DO control module. The key bioprocess parameters maintained during

fermentation included agitation (300-500 rpm), pH (setpoint 4.8) was adjusted with 28% NH_4OH and 50% H_3PO_4 and temperature (28°C). Polypropylene glycol was used as antifoam to prevent excessive foaming.

Assay of endoinulinase, SDS PAGE and PAS staining : The reaction mixture of 600 μl of 5% (w/v) inulin dissolved in 50 mM sodium acetate buffer (pH 6.0), 10 μl of 0.5 M CaCl_2 , and 150 μl of appropriately diluted enzyme. The reaction was then incubated at 55°C for 10 min and ended by incubating the reaction mixture in boiling water for 5 min. The amount of reducing sugar liberated was determined using DNS method, where absorbance was measured at 520 nm (Miller, 1959). Endoinulinase activity was directly proportional to μmol of reducing sugar liberated per minute under the assay conditions (Chen *et al.*, 2009). SDS-PAGE was performed on a 12% polyacrylamide gel and the gel was developed with 0.25% Coomassie Brilliant Blue R-250. For the detection of glycosylation by PAS staining, the protein was separated on 12% SDS-PAGE gel, the glycoprotein was oxidized with 1% periodic acid for one hour. The protein band was stained

with Schiff's reagent and incubated in dark. The reaction was finally terminated with 0.1% sodium metabisulfide solution.

Characterization of recombinant endoinulinase : The relative activity was determined at diverse pH values ranging from 4.0 to 8.0 at 60°C. To estimate pH stability, samples were incubated at 60°C for 1 hr with respective pH buffers and checked for residual activity. The optimal temperature of recombinant endoinulinase was checked by analyzing the enzyme activity at diverse temperatures 30-80°C in sodium acetate buffer, pH 6.0. The stability of enzyme with respect to temperature was intended by incubating endoinulinase at varied temperatures up to 1 hr at pH 6.0. The K_m and V_{max} values were calculated using line weaver-Burk plot.

TLC and HPLC analysis : Thin-layer chromatography (TLC) was carried out for the hydrolysed product on silica gel 60 TLC plate. The solvent system of n-butanol-ethanol-water (5:3:2 v/v/v) was used for the capillary movement and the sugar spots were developed with 5% sulfuric acid in methanol and the plate was

Fig. 1: (A) Schematic representation of recombinant plasmid expression cassette; (B) Schematic diagram for cloning and expression of *A. niger* endoinulinase in *Pichia pastoris*; (C) Genomic DNA PCR analysis. Lane 1 pPICZB-*INU1* plasmid PCR product as a positive control. Lane 2 DNA 1kb marker. Lane 3-11. PCR product of positive recombinant transformants. Lane 12. Negative control (only plasmid); (D) Shake flask expression profile of positive clones; (E) SDS-PAGE examination of recombinant endoinulinase expressed. Lane 1. Protein molecular marker. Lane 2, uninduced culture supernatant. Lane 3-9, culture supernatant of recombinant *Pichia pastoris* GS115- after induction by methanol every 24 hrs. Protein band with approximately 70 kDa molecular weight was evidently apparent on the SDS-PAGE. PAS staining of recombinant endoinulinase, Lane 1. Protein molecular weight marker Lane 2. PAS staining for glycosylated recombinant endoinulinase.

kept at 100°C for 3 min (Bernardi *et al.*, 2015). HPLC analysis was performed for the product of inulin hydrolysis by using a Shodex Asahipak NH2P-50 4E column (4.6 × 250 mm, Shodex Separation & HPLC Group, Japan) and an RI (refractive index) detector. The elution buffer contained the mixture of 75% acetonitrile and 25% water, and the flow rate was of 0.6 ml min⁻¹ was maintained at 25°C.

In-vitro fermentation of IOS by lactic acid and bifidogenic bacteria : To confirm growth was dependent on IOS utilization, lactic acid and bifidogenic bacterial strains were inoculated into 10 ml MRS basal broth containing 2% glucose (positive control), 2% commercial FOS and 2% IOS (recombinant-endoinulinase hydrolsed) as carbohydrate source in a 25 ml flask for 24 hrs at 37°C and 200 rpm. (modified the protocol as per Rossi *et al.*, 2005) The final cell density of probiotic bacteria for utilization of prebiotics was measured by Optical density at 600 nm after 24 hrs.

Results and Discussion

The constructed expression vector *pPICZαB-INU1* shown in the schematic diagram (Fig. 1B), harbors the 1482-bp (comprises 494 amino acid) *INU1* gene sequence in frame with the alpha-factor pre-pro secretion signal between the AOX1 promoter and AOX1 transcriptional terminator shown in Fig 1A. The pre-pro-sequence of *S. cerevisiae* provides extracellular secretion of several heterologous expressed proteins in *P. pastoris* (Ghazi *et al.*, 2006; Yun *et al.*, 1997). The positive transformants were confirmed, by genomic DNA PCR using gene-specific forward and reverse primers. Integration of

recombinant plasmid construct in *Pichia* genome was confirmed by the presence of 1.5 kb band in 1% agarose gel electrophoresis (Fig. 1C) and positive clones identified through genomic DNA PCR were selected for shake flask studies. The gene homology examination was carried out by comparing *A. niger* (DSM 2466) with *A. ficuum* JNSP5-06, *A. niger* CBS513.88 and *A. niger* 9891 that resulted in more than 96% endoinulinase gene similarity.

The extracellular expression of endoinulinase by positive transformants was studied in expression media containing methanol, which was used for the overexpression of endoinulinase gene through induction of AOX promoter sequence. The level of recombinant protein expression was enhanced by well controlled and powerful AOX promoter operated by methanol (Vogl and Glieder, 2013). The recombinant endoinulinase expressed successfully into the expression media and maximum endoinulinase activity observed after 5 days in shake flask level was 33 U ml⁻¹ (Fig.1D), which was 2 fold higher when compared to endoinulinase expression by wild-type *A. niger*. SDS-PAGE analysis showed a band of monomeric glycoprotein with a molecular size range of 70 kDa (Fig.1 E).

The heterologous protein expression in *P. pastoris* underwent post-translation glycosylation modification as per the glycosylation machinery of eukaryote, additional carbohydrate moieties were attached to the core protein and also varies among the amino acid sequence of interest (Moura *et al.*, 2007). The molecular weight of endoinulinase was increased because of the addition of carbohydrate moieties in the potential glycosylation sites present in the gene sequence. To determine the

Fig. 2 : Fermentation profile of recombinant endoinulinase produced through conventional approach at 28°C. The conventional approach fed-batch cultivation which was normally carried out at 28°C resulted in maximum endoinulinase expression of 65.7 U ml⁻¹. The data are mean ± SD of three independent analysed values. Blue color indicates OD @ 600 nm and red color indicates U ml⁻¹.

accumulation of carbohydrate moieties linked with recombinant endoinulinase, PAS staining was performed to confirm the glycosylation of protein as shown in Fig.1E. Overall molecular weight of recombinant endoinulinase was approximately 70 kDa, in that approximately 30–35% was contributed by the carbohydrate attachments. To increase the recombinant endoinulinase expression significantly, fed batch cultivation with the conventional and temperature limitation approaches were explored. *Aspergillus fumigates* endoinulinase gene was overexpressed in *Pichia* spp. and successfully produced the enzyme as a result of conventional fermentation process (Chen *et al.*, 2015).

In the current study, the conventional approach fed-batch cultivation which was normally carried out at 28°C, resulted in maximum endoinulinase expression of 65.7 U ml⁻¹ (Fig. 2) and to further improve the expression of recombinant endoinulinase temperature limitation approach at 20°C during induction phase was studied and resulted in 82.5 U ml⁻¹ after 120 hrs of induction phase (data not shown). The shift to a lower temperature during methanol induction decreased the dead cell count and resulted in

less proteolysis which was directly correlated to an enhanced expression profile of recombinant proteins (Jahic *et al.*, 2003). In the current study shift to a temperature significantly enhanced the productivity of endoinulinase compared to conventional approach. The endoinulinase gene isolated from wild-type *Arthrobacter* spp. S37 strain was engineered and expressed in *Yarrowia* spp. The maximum activity reported by positive recombinant clone was 16.7 U ml⁻¹ (Montesino *et al.*, 1998). *A. niger* 9819 endoinulinase gene was cloned and overexpressed intracellularly in *P. pastoris* GS115 (Wang *et al.*, 2004). But in this study, the endoinulinase was actively secreted extracellularly which reduced the need for further purification steps. The wild-type *A. niger* strain used in this work was found to overexpress endoinulinase when compared to different endoinulinase producing fungal strains and it was found that the strain produced only endoinulinase (Gern *et al.*, 2001). In the current study, the specific isolation of endoinulinase gene was performed, whereas many *Aspergillus* strains produce both endoinulinase and exoinulinase which made isolation of specific gene of interest difficult.

Fig. 3 : (A) The optimum pH for endoinulinase activity was pH 6.0; (B) The optimum temperature for endoinulinase activity was studied, the optimal temperature to obtain maximum activity was 60°C; (C) The stability of pH was measured and shown to have stability in varied pH range; (D) The stability was analysed by incubating recombinant endoinulinase, the enzyme retained about 85% of the activity at 60°C. Values are mean of three replicates $\pm$ SD.

Fig. 4 : (A). HPLC profile for inulin hydrolysis by recombinant endoinulinase. Analysis shows peaks for Inulotriose (DP3) with retention time of 12 mins, inulotetraose (DP4) with retention time of 14 mins; (B) TLC profile for inulin hydrolysis by recombinant endoinulinase; (C) IOS which is obtained by hydrolyzing inulin by recombinant endoinulinase enzyme show prebiotic efficacy in supporting the growth of *Bifidobacterium* and lactic acid bacteria. Probiotic microorganism growth in presence of MRS-IOs comparatively similar with growth in MRS supplemented with glucose and commercial FOS.

The optimum pH for endoinulinase activity was studied, more than 80% residual activity of the enzyme was retained at pH 6.0 (Fig. 3A). The pH stability was measured by analyzing the remaining enzyme activity after incubating recombinant endoinulinase at various pH for 1 hr at 60°C. It was stable over a pH choice of 4.0-8.0, retaining its initial activity over 40% at acidic pH and 98% at optimum 6.0 pH (Fig. 3B). Optimum pH for yeast strains producing endoinulinases was found to be 4.4 to 6.5 (Rastall, 2010). The optimal temperature for endoinulinase activity was studied by incubating the recombinant enzyme in a temperature ranged from 30-80°C at pH 6.0 and the optimal temperature to obtain maximum activity was 60°C (Fig. 3C). The temperature stability was analyzed by incubating endoinulinase

at different temperatures and the enzyme retained about 85% of the activity at 60°C (Fig. 3D). K_m of recombinant endoinulinase was found to be 20 mM while its V_{max} was 111 U l⁻¹ min⁻¹. The recombinant enzyme showed absolute specificity towards inulin substrate and produced inulotriose (DP3) and inulotetraose (DP4) as hydrolyzed product which was shown in thin layer chromatography (Fig. 4B) and the peaks for DP3 and DP4 was evident in HPLC analysis (Fig. 4A). Long-chain fructans (DP range greater than 9) was formed by using inulin as a substrate, while short-chain fructans were produced (DP lesser than 4) by using sucrose as substrate (Roberfroid *et al.*, 1998). The recombinant endoinulinase showed sucrose hydrolysis pattern, with DP range (DP < 4) for inulin hydrolyzed product, however the

hydrolyzed product showed similarity with commercial prebiotics like GOS® (DP=2–4) and NutraFlora® P-95 (DP=2–4).

IOS which was obtained by hydrolyzing inulin by recombinant endoinulinase enzyme showed prebiotic efficacy in supporting the growth of *Bifidobacterium* and lactic acid bacteria (Fig. 4C). Growth rates of probiotic bacteria utilizing IOS, as a carbon source, were similar to growth observed when using glucose and commercial FOS as a carbon source. These results recommended that IOS utilization did not require an induction period and that IOS was an equally good substrate as glucose in sustaining the growth of probiotics. The short chain IOS derived as an end result of the current study can be used as potential prebiotics, since nearly all probiotic bacteria ferment shortchain FOSs for their growth promotion (Rossi *et al.*, 2005)

The endoinulinase gene of 1482 bp from *Aspergillus niger* was genetically engineered in the GS115 host and was secreted extracellularly using α signal sequence. The recombinant endoinulinase produced was further characterized. The IOS produced using recombinant endoinulinase from *Pichia pastoris* showed sustained growth of probiotics. The prebiotics produced after hydrolysis by recombinant endoinulinase can be used in aquaculture and food industries.

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