Non-conventional expression of recombinant chitinase A originating from *Bacillus licheniformis* DSM8785, in *Saccharomyces cerevisiae* INVSc1

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Abstract: Chitinases are glycosyl hydrolases, that cleave the β-1,4 linkage between *N*-acetyl glucosamines present in chitin chains. Chitin is the second most abundant polysaccharide on Earth after cellulose, and it is produced in the exoskeleton of crustaceans and insects, and in some parts of the cell walls of fungi. Enzymatic development and the extraction of superior derivatives from chitin wastes – such as chitooligosaccharides with vast importance in the medical and biofuels industry – lead to the necessity of creating chitinases using different strains of organisms. In this paper, the chiA gene from the *Bacillus licheniformis* DSM8785 encoding chitinase A (ChiA) with C-terminal hexahistidine tag was cloned and expressed in the extracellular expression system pYES2 from *Saccharomyces cerevisiae* INVSc1 as a hyperglycosylated enzyme. The production of recombinant ChiA was successfully confirmed by dot blotting, using anti-His antibodies. The optimal time of expression was identified to be 24 h when galactose was added only at the beginning of fermentation, the chitinase activity starting to decrease after this threshold. Nevertheless, in another experiment, when galactose was added every 24 h for 72 h, the expression continued for the entire period. The purified enzyme was detected, using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), as a heterogeneous diffuse band between 80 and 180 kDa. The molecular mass of the same ChiA enzyme expressed in *Pichia pastoris* KM71H and *Escherichia coli* BL21 (DE3) was compared using SDS-PAGE with ChiA expressed in *S. cerevisiae* INVSc1. The activity of ChiA was determined using the fluorogenic substrate, 4-methylumbelliferyl β-D-*N*,*N*,*N*-triacetylchitotrioside (4MUTC). Using a bioinformatics simulation, the number of the glycolsylation sites of the ChiA gene sequence and the proximity of these sites to the alpha factor sequ-

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ence were hypothesized to be a possible reason for which ChiA enzyme was internally expressed.

Keywords: chitinolytic enzymes; molecular cloning; dot blotting, fluorescent assay; glycosylation.

INTRODUCTION

Chitinases (EC 3.2.2.14) are glycosyl hydrolases that hydrolyze the *β*-1,4 linkage of the *N*-acetyl glucosamine group in chitin chains.[1] The production of chitinases is an important step in the bioconversion process of treating shellfish waste, resulting in proteins for animal and aquaculture feed and in valuable chito-oligomers. The production of chitinase enzymes is presently unprofitable due to the high prices of commercially available chitinases. A more efficient and economically reliable process is essential for chitin exploitation and the management of shellfish wastes.[2] Genetic engineering technology offers a method to approach this problem by using recombinant enzymes. Recombinant enzymes, which are used in biotechnology or in waste management, require high thermostability. The glycosylated proteins are more stable at higher temperatures compared to their non-glycosylated counterparts. As a general rule, when proteins are expressed by *Escherichia coli* and other types of bacteria, a glycosylation portion is not added to the protein. For eukaryotic cells, glycosylation constituents are usually added to the expressed proteins, helping secretion outside the cells. The glycosylation pattern is species dependent. For example, *Saccharomyces cerevisiae* usually creates a hyperglycosylation pattern that depends on the primary structure of the protein, while *Pichia pastoris* creates a rather frequent and repetitive glycosylation pattern, similar to all types of expressed proteins.[3] While glycosylation of *P. pastoris* can have 20 residues in length, *S. cerevisiae* exceeds 100 residues.

It is well known that glycosylated proteins and enzymes are more thermostable than less or aglycosylated variants, therefore strains with glycosylation pathways are often preferred for production of proteins. One of the most well-known strains that can create hyperglycosylated enzymes is *S. cerevisiae*. In contrast with other yeasts, such as *P. pastoris*, the endoplasmic reticulum (ER) of *S. cerevisiae* involved in the glycosylation process is dispersed differently in the cells.[4] This feature makes *S. cerevisiae* a model organism with regard to its mode of glycosylation. This yeast is also an attractive host for the production of recombinant proteins, enzymes and different pharmaceuticals.[5] Other advantages offered by these systems are well-defined DNA transformation and secretory system (the ability to secrete biologically active enzymes into the culture medium), rapid growth, and the simple and inexpensive culture media.[6] It is therefore a very attractive system for the production of industrial enzymes, such as chitinase, *α*-amylase, xylanase, *β*-glucanase and human therapeutic proteins.[7]

In the present paper, the chitinase A gene from *Bacillus licheniformis* DSM8785 was cloned and expressed in *S. cerevisiae* INVSc1. The optimal time of expression was investigated when galactose was added only at the beginning of induction or every day during fermentation. The ChiA was purified from *S. cerevisiae* INVSc1 cells and its molecular mass was compared to the same ChiA expressed by *P. pastoris* KM71H and *E. coli* BL21 (DE3).

EXPERIMENTAL

Chemicals, enzymes and antibodies

The chemicals were purchased from Carl Roth and Sigma-Aldrich, Germany. The restriction enzymes Kpn I and Bam HI, T4 DNA ligase, Dpn I; calf intestinal alkaline phosphatase (CIAP) and PNGase F kit were purchased from Thermo Fisher Scientific or New England Biolabs, Ipswich, MA, USA. The DNA purification kit, plasmid purification columns (NucleoSpin®) and PCR products were supplied by Macherey-Nagel, Germany. Pfu HF DNA polymerase and Taq DNA polymerase were bought from Agilent Technology, Santa Clara, CA, USA. Anti-6-His antibody produced in rabbit, goat anti-rabbit IgG antibody, (H+L) alkaline phosphatase conjugate and chromogenic phosphatase substrate solution (Nitro-Blue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-indolylphosphate toluidine salt (BCIP)) were purchased from Sigma Aldrich.

Plasmids and genes

The synthetic ChiA gene from *Bacillus licheniformis* DSM8785 (GenBank Accession Number FJ465148) was provided by GenScript (USA) and used as a template for molecular cloning in the yeast expression system. The *S. cerevisiae* INVSc1 expression vector pYES2 (#V82520) was purchased from Invitrogen, USA. The ChiA gene was cloned as well in *Pichia pastoris* KM71H pPICZαA extracellular expression system[8] and *E. coli* BL21 (DE3) pET22b(+) periplasmic expression system (this research is the subject of another article).

Organisms and growth conditions

S. cerevisiae INVSc1 (#C81000, Invitrogen) was cultured in SC-U medium containing 2 vol. % glucose at 27 °C. The basal components of the SC-U medium were 0.67 % yeast nitrogen base, 0.12% amino acid mix without uracil (0.01 % adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan; 0.005 % aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, valine), 2 vol. % glucose or galactose. The SC-U medium and glucose solution were autoclaved at 120 °C for 20 min. The galactose solution was added to the medium in order to induce enzyme expression and was separately sterilized using a 0.2 μm PDVF filter. For the preparation of the solid medium, in addition, 2 % agar was added before autoclaving. After the yeast was transformed with ChiA_pYES2 or pYES2 plasmid, the cells were cultured on YPD medium containing chloramphenicol (50 μg mL^{-1}). Plasmids were also inserted and amplified into *E. coli* XL10 Gold ultra-competent cells and grown in LB medium.

Subcloning of ChiA gene into pYES2 expression vector

The ChiA gene was cloned by the classical PCR/restriction method using forward primer FP pro α Kpn I (5′ ATC GGT ACC ATG AGA TTT CCT TCA ATT TTT ACT GCT GTT TTA TTC-3′) and reverse primer RP his Bam HI (5′-ATT GGA TCC TCA GTG GTG GTG GTG GTG GTG TTC GCA GCC TCC GAT CAG CC-3′). The PCR reaction mixture consisted of 1 μL ChiA template (58.6 ng μL^{-1}), 2 μL forward primer (25 μM), 2 μL reverse pri-

mer (25 µM), 2 µL deoxynucleotide mixture (dNTP, 10 mM), 10 µL 10x Pfu buffer, 2.5 µL Pfu DNA polymerase (2.5 U/µL) and 80.5 µL ultrapure water. PCR amplification comprised an initial denaturation step at 95 °C for 3 min followed by 25 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 4 min, followed by a final extension step at 72 °C for 10 min. The reaction products (100 µL) were treated with 1 µL Dpn I (10 U µL^{-1}) for 1 h at 37 °C to eliminate the template, then purified on NucleoSpin Plasmid Columns.

The pYES2 vector and PCR products containing amplified ChiA were each double digested with Kpn I and Bam HI. The vector was treated with calf intestinal alkaline phosphatase and the components were ligated overnight at 17 °C using T4 DNA ligase. The ligated vector was inserted into *E. coli* XL10 Gold cells by heat-shock transformation.[9] The insert was verified by colony PCR and Sanger sequencing.[10] The verified ChiA_pYES2 and pYES2 plasmids were used to transform the *Saccharomyces cerevisiae* INVSc1 strain, using the lithium acetate/single-strand carrier DNA/poly(ethylene glycol) method.[11]

Chitin agar plate assay

Around 15 colonies of transformed *S. cerevisiae* INVSc1 containing ChiA_pYES2 or pYES2 were transferred on plates with a solid SC-U media containing 2 % galactose and 0.5 % colloidal chitin. The plates were incubated at 27 °C for 3 days. After this step, the *S. cerevisiae* INVSc1 cells were removed by washing the plate surface with water and the plate was subjected to staining with Congo Red.[8]

Dot blot analysis

A preculture (25 µL) of *S cerevisiae* INVSc1 containing ChiA-pYES2 or pYES2 was inoculated in an Elisa plate containing 85 µL well of SC-U with galactose and then the plate was incubated at 30 °C and 900 rpm for 48 h. The fermented cultures (18 µL) were transferred to a dot blot plate fitted with a nitrocellulose membrane and connected to a vacuum. After each 3 µL transferred in 6 rounds, the cultures were allowed to adsorb for a few minutes. Anti-6-His antibody produced in rabbit was used as the positive control. The nitrocellulose membrane was incubated for 30 min in 25 mL 5 % milk powder. The membrane was washed several times with PBS-T buffer. The anti-6-His antibody produced in rabbit was added (3 µL of 1 mg mL^{-1} antibody in 10 mL PBS-T) and incubated for 1 h under mild mixing. The membrane was washed several times and then the goat anti-rabbit IgG antibody, (H+L) alkaline phosphatase conjugate (3 µL of 0.6 mg mL^{-1} antibody in 10 mL PBS-T) was added. After 1 h, the second antibody was removed by washing with PBS-T. Then, 10 mL of NBT/BCIP (100 µL NBT (18.8 mg mL^{-1})/BCIP (9.4 mg mL^{-1})) in 67 % DMSO stock solution diluted in 10 mL alkaline phosphatase buffer) was added. After 5 min, the dots were observed and photographed.

Heterologous expression of ChiA

A volume of 6 mL of *S. cerevisiae* INVSc1 preculture containing the recombinant plasmid ChiA-pYES2 was diluted in 50 mL of SC-U medium supplemented with 2 % galactose contained in two different flasks. The cultures were incubated for 72 h at 27 °C and 160 rpm. In one culture, galactose was added at the beginning of the expression, in the other culture, 2.5 mL of 20 % galactose was added every 24 h, for 72 h. Cell culture samples (1.5 mL) were taken after 0, 4, 24, 48 and 72 h of expression. The samples were kept at –20 °C until use. Aliquots from samples (1 mL) were centrifuged at 11000 g for 5 min and the supernatant was separated from the cells in order to determine whether the expression of chitinase was intra- or extracellular. The cells were diluted with 1 mL of water to equalize the cell concentration to the supernatant concentration.

Chitinase activity assay

Chitinase activity was verified by fluorogenic substrate analysis using 4MUTC as follows: 25 μL of samples were rapidly mixed with 25 μL of 0.05 mg mL^{-1} 4MUTC and the fluorescence product was measured at an excitation of 355 nm and emission of 460 nm. The negative control comprised 25 μL distilled water mixed with 25 μL of 0.05 mg mL^{-1} 4MUTC.

Production of ChiA

A preculture of *S. cerevisiae* INVSc1 containing ChiA-pYES2 plasmid (5 mL) was diluted in 400 mL of SC-U medium supplemented with 2 % glucose (*OD* 600 start = 0.083). The culture was divided into two portions of 200 mL in two Erlenmeyer flasks (5 L). The cultures were incubated at 27 °C, 160 rpm for 24 h. After 24 h, the *OD* 600 of the culture was approximately 1.805. Chitinase expression was induced by the addition of 1 L of SC-U supplemented with 2 % galactose (six times dilution, *OD* 600 start = 0.608), to each 200 mL culture. The culture was incubated for 24 h at 27 °C, 160 rpm. After 24 h of incubation at 27 °C, the expression culture was centrifuged at 6200g, 4 °C for 20 min. The cells were resuspended in 70 mL of their own supernatant then passed through a French press four times at 10000 psi* (pound-force per square inch). After pressing, the cells were centrifuged again for 30 min at 4 °C at 27200*g*. The enzyme-containing supernatant was first filtered through a 0.2 μm membrane to remove cell debris and then filtered through centrifugation at 3000*g* using Millipore 50 kDa membrane filters. The concentrated enzyme was dialyzed in 4 L of 10 mM Tris-HCl buffer, pH 7.0, for 24 h at 4 °C.

Purification of ChiA

A sample of dialyzed enzyme (11 mL) was injected into a 20 mL DEAE Sepharose FF 16/10 HiPrep column. The column was equilibrated with 2 column volumes of 10 mM Tris--HCl buffer, pH 7.0. The elution gradient was operated from 0 to 100 % of 10 mM Tris-HCl/1 M NaCl buffer pH 7.0, over 20 column volumes. The injection flow and flow rate were 0.5 and 1 mL min^{-1}, respectively. The volume of each collected fraction was 2 mL.

The concentration of purified chitinase enzyme was determined using a spectrophotometric method at 280 nm adapted after Grimsley and Pace.[12] A solution of commercial chitinase from *Streptomyces griseus* with a known concentration was used as the standard.

Deglycosylation of purified ChiA

The deglycosylation reaction comprised of 40 μL ChiA (1.2 μg μL^{-1}) and 20 μL glycoprotein denaturing buffer was first incubated at 100 °C, after which 10 μL glycobuffer 2, 10 μL NP-40 (10 %), 3 μL PNGase F and 17 μL distilled water were added. The reaction mixture was incubated at 37 °C on a shaker platform. The same procedure was undertaken for the negative control, except for the presence of PNGase F.

SDS-PAGE analysis

Three different samples of the same purified recombinant ChiA produced in three different strains of *S. cerevisiae* INVSc1, *P. pastoris* KM71H[8], *. coli* BL21 (DE3, the purification of this strain will be the subject of another article) was exposed to polyacrylamide gel electrophoresis (PAGE) according to Laemmli, 1970.[13] Purified ChiA samples (30 μL, 1.0 mg mL^{-1}) were loaded into the gel. The samples were subjected to electrophoresis using a current of 120 V for 90 min. The proteins were stained with Comassie Brilliant Blue, then progressively

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* 1 psi = 6896 Pa

decolorized with 10 % acetic acid solution. The deglycosylated sample was subjected to SDS--PAGE under the same conditions.

Bioinformatics analysis

For bioinformatic analysis, the DNA sequence of the ChiA was first translated in protein sequence using the Translate tool - Expasy. Based on the amino acids sequence, the theoretical molecular mass and isoelectric pH were calculated applying the Compute pI/Mw tool – Expasy. The amino acids sequence (Fasta format) was analyzed in a NetNGlyc - 1.0 server in order to predict the *N*-glycosylation sites and examine the sequence context of Asn-Xaa-Ser//Thr sequins, where Xaa is represented by any amino acid except for proline.[14] According to this bioinformatics protocol, the protein sequences of hexose oxidase (HOx), cellobiose dehydrogenase (CBDH), and glucose oxidase (GOx) were compared to chitinase A. The comparison was focused on the number of *N*-glycosylation points in the protein structures, and the distances between these points and *N*-glycosylation points from signal peptides attached to proteins. The results obtained were outlined in a schematic figure.

RESULTS AND DISCUSSIONS

Molecular cloning of chiA gene in pYES2 expression vector

The ChiA gene derived from *Bacillus licheniformis* DSM8785 was cloned into the extracellular expression vector pYES2, compatible with the expression system of *S. cerevisiae* INVSc1. The pYES2 vector is an extracellular expression vector that allows the cloning of the gene of interest and the selection of transformants on uracil deficient environments due to its *ura*3 gene construct for uracil-specific synthesis required for cell growth.[15] The *B. licheniformis* DSM8785 ChiA gene has an open reading frame (ORF) of 2023 bp, including *α* factor and 6x *his* tag sequences. The main genetic elements involved in gene cloning and expression are outlined in Fig. 1A. The presence of the GAL1 promoter induces the expression of chitinase in *S. cerevisiae* in the presence of galactose and acts as a repressor in the presence of glucose. For the extracellular expression of chitinase, the gene was cloned with the *α*-factor pro-peptide sequence.[16] The sequence encoding 6 histidines was introduced at the C end of the gene that allowed easy detection of recombinant chitinase A by dot blot analysis. Restriction endonucleases Bam HI and Kpn I were used in the gene and vector digestion step to create compatible sticky ends. The ChiA_pYES2 recombinant plasmid scheme was created using Vector NTI bioinformatics software, created by InforMax Inc., North Bethesda, MD, USA. The ChiA gene linked to 6 histidines sequence encodes an enzyme with a theoretical molecular mass of 64.77 kDa and an isoelectric pH in the acid range at 5.21, according to the Expasy Bioinformatics Resource Portal program.[17]

PCR amplification of the gene consisted of introducing restriction sites for Bam HI and Kpn I endonucleases at its ends, for ligation compatibility in the pYES2 vector. The ChiA gene amplified by PCR (Fig. 1B, line 1 and the vector pYES2 line 2) were digested with Bam HI and Kpn I restriction enzymes and

ligated to form the recombinant plasmid ChiA_pYES2. The ligation products were checked on a agarose gel and, as can be seen in Fig. 1B, line 3, there are several DNA fragments. The DNA fragments located at about 2.0 and 6.0 kbp correspond to the ChiA gene and pYES2, respectively, representing non-ligated fragments. The 8.0 kbp DNA fragment represents the recombinant ChiA_pYES2 plasmid, summing the vector and gene mass. At approximately 4.0 kbp, a band appears that could represent self-ligation of two ChiA genes, a situation created when several gene fragments are partially digested by one of the restriction enzymes, or not digested at all. By DNA sequencing, the correct insertion of the ChiA gene into the vector pYES2 was confirmed.[10]
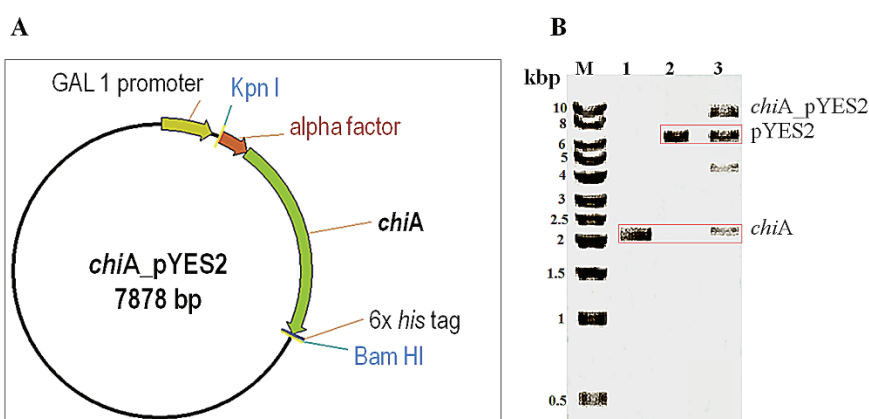
Fig. 1. Theoretical scheme of ChiA gene inserted into pYES2 vector and the main genetic elements used for cloning and expression (A). Agarose gel electrophoresis for the ChiA gene (B). Line M = DNA molecular marker, line 1 = PCR amplified ChiA gene, line 2 = pYES2 vector, line 3 = ligation products.

A fast chitin agar plate assay highlighted that there was no difference between cells that produce recombinant chitinase and those that did not produce (data not shown). The information obtained from this test is proof of chitinase activity. The negative control shows enzymatic activity coming from native chitinase in *S. cerevisiae*[18] located in the periplasmic space and allowing the enzyme to act on the secreted chitin in that space during cell septum formation. The function of native chitinase in cell division is suggested by the high concentration found during exponential growth, compared to the stationary phase, in yeast cells.[19] Additional experiments were performed to detect recombinant chitinase A. The ChiA gene was inserted into the expression vector pYES2 having six histidine residues at the C terminus, which allowed western blotting to detect it using rabbit anti-His antibody and goat anti-rabbit antibody coupled to alkaline phosphatase. Thus, a dot blot analysis was performed using samples from a culture of *S. cerevisiae* INVSc1 that expressed chitinase A. The negative control

consisted of yeast cells containing only the vector and not producing recombinant chitinase, and the positive control consisted of the rabbit anti-His antibody.

Following this assay, the presence of recombinant ChiA in the yeast culture containing the plasmid ChiA_pYES2 was demonstrated, as could be seen in Fig. 2 (lines 1 and 2). In the negative sample (line N) containing *S. cerevisiae* cells with pYES2, the peptide sequence of 6 histidines was not detected.
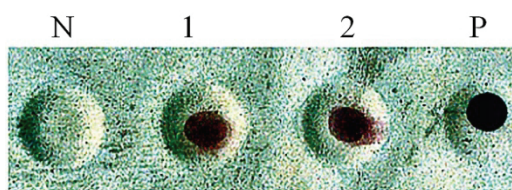

Fig 2. Dot blot analysis for recombinant chitinase expressed by *S. cerevisiae* INVSc1 cells. Line N = negative control – *S. cerevisiae* INVSc1 pYES2 cells, line 1–2 = *S. cerevisiae* INVSc1 ChiA_pYES2 cells, line P = positive control – rabbit anti His antibody.

Optimization of protein expression

Since each enzyme behaves differently, it is important to optimize the growth and expression conditions. The recombinant *B. licheniformis* ChiA gene was controlled by the GAL1 promoter in *S. cerevisiae* allowing the induction of gene expression on 2 % galactose. The coding sequence was also fused to the α--factor pro-peptide to ensure the secretion of the enzyme to the extracellular medium. The expression of ChiA in the *S. cerevisiae* strain INVSc1 was analyzed by growing the strain transformed with ChiA_pYES2 in a small volume of SC-U expression medium, and the culture samples were tested for the presence of ChiA over the next 72 h. This analysis was realized within two conditions: first, with the addition of galactose inductor only at the moment of the beginning of expression and second, with the addition of galactose every 24 h, for 72 h. The optimal chitinase expression time was found to be about 24 h after galactose induction. After 48 h, the relative activity of chitinase droped below 20 % (Fig. 3A). Surprisingly, when galactose was added to the expression medium on a daily basis, chitinase activity existed throughout the expression range (0–72 h), which translates to the fact that the recombinant enzyme was synthesized constantly (Fig. 3B). The consumption of galactose is the explanation for which the production of ChiA stagnates after 48 h, this fact being revealed by a decrease of the enzyme activity.

To determine whether recombinant chitinase A is produced as an intra- or extracellular enzyme, samples were taken after 24 h of expression and centrifuged to separate the supernatant from cells. Using 4MUTC fluorogenic substrate, the chitinase activity from the supernatant and cells of *S. cerevisiae* con-

taining ChiA_pYES2 or pYES2, was verified. Unexpectedly, only in the cell samples was chitinase activity observed, as could be seen in Fig. 4.
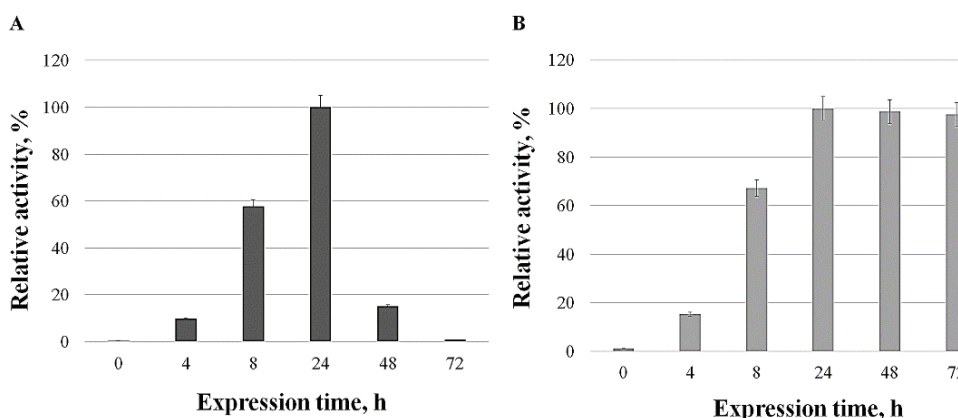

Fig. 3. Optimal expression time of recombinant chitinase A produced by *S. cerevisiae* INVSc1, on a 4 MUTC fluorogenic substrate, at different expression time intervals. A) Galactose was added only at the beginning of expression; B) galactose was added at every 24 h, for 72 h. Fluorescent assay was realized on culture samples.
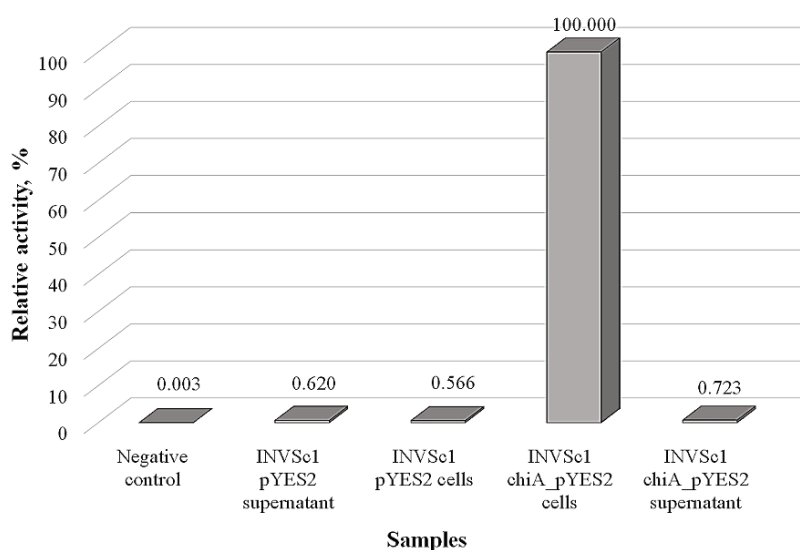

Fig. 4. Relative activity of recombinant chitinase from supernatant and cells of *S. cerevisiae* INVSc1, on a 4MUTC fluorogenic substrate. The negative control contained only distilled water and fluorogenic substrate.

Based on the optimized small-scale fermentation conditions, the recombinant ChiA was produced for 24 h at 27 °C. The enzyme was recovered from the cells and purified using anion exchange chromatography on a Sepharose/DEAE column.

From the fractions obtained, only those that showed chitinase activity on the fluorogenic substrate were collected. Purified recombinant chitinase A revealed on SDS protein electrophoresis to be a highly glycosylated enzyme.

In order to see the mode of expression, from a glycosylation point of view, ChiA produced in *S. cerevisiae* INVSc1 was compared to the same enzyme produced in *P. pastoris* KM71H[8] and *E. coli* BL21 (DE3) (purification of ChiA from this strain will be subject of another research article). As could be observed in Fig 5A, line 1, recombinant ChiA produced in *S. cerevisiae* is represented by a heterogeneous diffuse band between 80 and 180 kDa, which means strong glycosylation. In Fig 5A, line 2, ChiA produced in *P. pastoris* shows a visible lower molecular mass, being situated between 70–130 kDa. In contrast, in line 3, from the same figure, ChiA produced in *E. coli* is aglycosylated, having a molecular mass around 75 kDa.
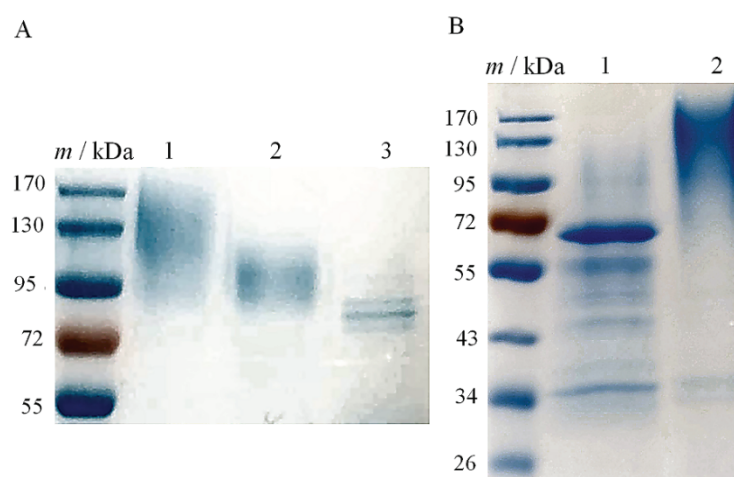

Fig. 5. SDS-PAGE (9 % gel) analysis of purified recombinant ChiA under reducing conditions. A) SDS-PAGE analysis of purified recombinant ChiA expressed by different strains. Line *m* / kDa = protein molecular marker, line 1 = ChiA expressed in *S. cerevisiae* INVSc1, line 2 = ChiA expressed in, line 3 = ChiA expressed in *E. coli* BL21 (DE3). B) SDS-PAGE analysis of deglycosylated ChiA produced in S. cerevisiae INVSc1. Line 1 = ChiA deglycosylated with PNGase F, line2 = ChiA hyperglycosylated.

Deglycosylation was performed using PNGase F, the most effective enzyme for removing completely N-linked oligosaccharides from glycoproteins,[20] in order to confirm the theoretical mass of ChiA. This reaction confirmed as well the hyperglycosylation of the recombinant ChiA in *S. cerevisiae* (Fig. 5B). In *S. cerevisiae* most proteins are synthesized in the extracellular medium as hyperglycosylated proteins.[21] Nevertheless, the recombinant ChiA, originated from *B. licheniformis* was not exported in extracellular space. In order to have a better

understanding on mode of expression of other proteins, in *S. cerevisiae* INVSc1, a synthetic view is presented in Table I. As could be seen, after expression, proteins have higher molecular mass, because of the glycosylation. Molecular mass of heterologous expressed proteins is even 3 times higher than mass of the same proteins produced in the organism of origin.

As it can be seen in Table I, extracellularly expressed proteins have relatively small mass even after glycosylation. Signal sequences are the strategic factors adjusting protein secretion. Introduction of a signal peptide improves the secretion of heterologous proteins in yeast. However, it is clear that even with α--factor signal peptide proteins can remain in the intracellular space. Some studies showed that the secretion efficiency of foreign proteins in recombinant microbes is strongly dependent on the combination of the signal peptides.[22,23] This could be a possible explanation for the case when some proteins are exported in extracellular space, some are not, when an α-factor signal peptide is used for secretion.

TABLE I. An overview of expression of different enzymes in *S. cerevisiae* INVSc1, by galactose induction

Organism of origin	Expression vector	Recomb. enzyme	m / kDa		Location of expressed enzyme	Peptide sequence	Ref.
			Native enzyme	Recomb. enzyme			
Bacillus licheniformis DSM8785	pYES2	Chitinase A	66.8	95–180	Intracellular	α-Factor signal peptide	This paper, 24
Melanocarpus albomyces	pMS174 pMS175	Laccase	80	95	Intracellular (major part) and extracellular	α-Factor signal peptide and propeptide	25
Aspergillus oryzae	pYES/EXL, pYES3/CT	Cutinase	26	–	Intracellular (cell walls and/or between cell wall and cell membrane) and extracellular (traces), extracellular for protoplasts	-	26
Lentinula edodes	pYES2	Cytochrome P450	46.8	61	Intracellular (microsomes)	–	27
Insect-derived amylolytic enzyme	pYES2	Alpha-amylase	–	~53	Intracellular and extracellular (major part)	No	22

TABLE I. Continued

Organism of origin	Expression vector	Recomb. enzyme	*m* / kDa		Location of expressed enzyme	Peptide sequence	Ref.
			Native enzyme	Recomb. enzyme			
Acinetobacter sp. SM04	pYES2-alpha (pYα)	Peroxiredoxin	20	–	Extracellular	α-Factor signal peptide	28
Aspergillus niger	pYES2	Xylanase	27.2	–	Extracellular	α-Factor signal peptide	29
Dioscoreophyllum cumminsii	pYES2	Monellin (sweet protein)	11	~10.7	Extracellular	α-Factor signal peptide	30
Bombyx mori	pYES2/CT	Cecropin antibacterial peptide	4	6–10	Extracellular	α-Factor signal peptide	31
Phanerochaete chrysosporium	pYES2	Cellobiose dehydrogenase	≈90	120–150	Extracellular	α-Factor signal peptide	32

In 1982, Elango *et al.*[19] during the transformation of yeast cells into protoplasts observed as well that only about half of the yeast chitinase were released into the medium, indicating that part of the enzyme is located in the periplasmic space, and the other part remains in vacuoles or intracellular vesicles.

The capacity of the endoplasmic reticulum (ER) to fold and process foreign proteins is a significant factor restricting the expression of foreign proteins in *S. cerevisiae* and could represent another reason for which ChiA remain blocked in the cells.

A schematic overview representing glycosylation sites of different enzymes expressed with a pro-peptide signal in *S. cerevisiae* pYES2 system is presented in Fig. 6. Chitinase A (Fig. 6A) and hexose oxidase Fig. 6B) enzymes even though they are cloned with α factor signal peptide are not externally expressed in the culture medium. Nevertheless, cellobiose dehydrogenase (Fig. 6C) and glucose oxidase (Fig. 6D) are secreted in the culture medium.

The main hypothesis in this case was correlated with the glycosylated sites and the molecular mass at which the enzyme reaches after glycosylation. Chitinase A was expressed in the *P. pastoris* KM71H pPICZαA system as an external glycosylated enzyme with a molecular mass between 70 and 130 kDa.[8] In this case, the signal peptide was efficient and transported the enzyme out of the cell. Despite this fact, in *S. cerevisiae* INVSc1, the signal peptide is not efficient, chitinase A being blocked in the intracellular or periplasmic space of the cells. In *S. cerevisiae* INVSc1, ChiA was internally expressed as a hyperglycosylated enzyme, with a molecular mass between 80 and 180 kDa, 50 kDa higher than of ChiA expressed in *P. pastoris*. Number of glycosylation residues at S. *cerevisiae*

is higher than at *P. pastoris* and this could be a possible explanation for blocking the ChiA in the cell.
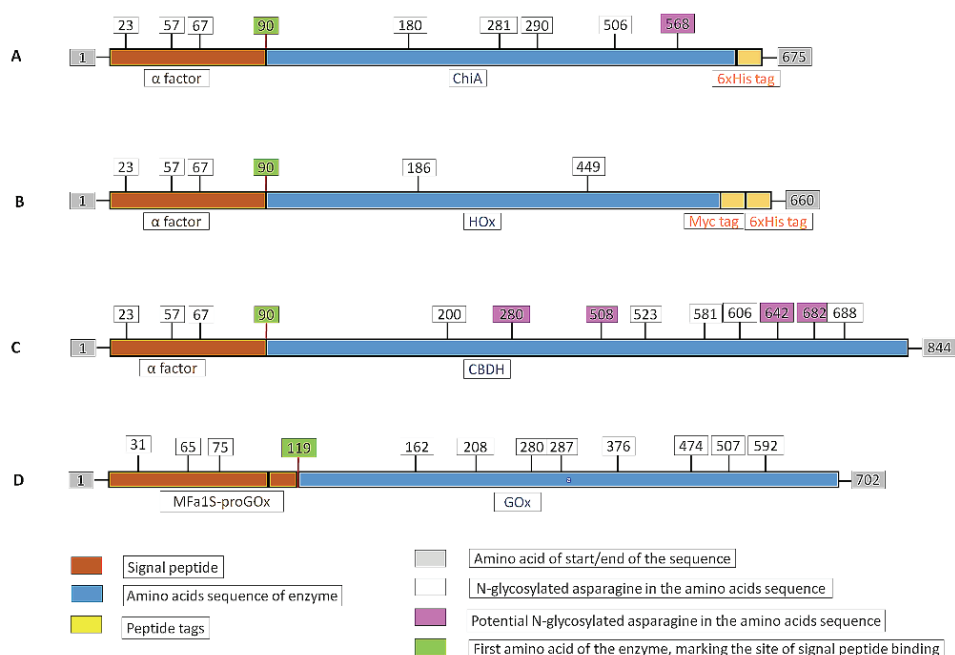

Fig. 6. Schematic representation of glycosylation sites in 4 different enzymes expressed in the *S. cerevisiae* pYES2 system. A) α factor_ChiA (chitinase A) 6xHis tag, B) *α*-factor_HOx (hexose oxidase) Myc_6xHis tags, C) *α*-factor_CBDH (cellobiose dehydrogenase), D) MFa1S-pro_GOx (glucose oxidase). The sequences were analyzed using Expasy Bioinformatics tools and NetNGlyc 1.0 Server – DTU.

CONCLUSIONS

The recombinant ChiA enzyme originating from *Bacillus licheniformis* has been successfully expressed in the *S. cerevisiae* INVSc1 expression platform. Unlike the native enzyme, the recombinant ChiA produced in *S. cerevisiae* INVSc1 was hyperglycosylated. Hyperglycosylation of ChiA was confirmed, as well, by a comparative analysis between the same recombinant ChiA produced in *P. pastoris* and *E. coli* systems. It was shown that by adding of galactose inductor, every day to culture medium, the expression of chitinase is constant, for 72 h. Production of hyperglycosylated ChiA into the yeast cells was confirmed by fluorescent activity assay. The number of the glycosylation sites of the ChiA gene sequence and the proximity of these sites to the α factor sequence were hypothesized to be a possible reason for which ChiA enzyme was internally expressed instead to be secreted by *S. cerevisiae* INVSc1 strain.

ИЗВОД

НЕКОНВЕНЦИОНАЛНА ЕКСПРЕСИЈА РЕКОМБИНАНТНЕ ХИТИНАЗЕ А ПОРЕКЛОМ ИЗ *Bacillus licheniformis* DSM8785 У *Saccharomyces cerevisiae* INVSC1

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Хитиназе су гликозил-хидролазе које цепају β-1,4 везу између *N*-ацетил-глукозамина, који су присутни у хитинским ланцима. Хитин је други најраспрострањенији полисахарид на земљи након целулозе и формира се у егзоскелету ракова и инсеката, а налази се и у неким деловима ћелијских зидова печурака. Ензимско дејство и екстракција виших деривата из хитинског отпада (као што су хитоолигосахариди који имају важну улогу у медицинској и индустрији биогорива) доводе до потражње за хитиназом и њеном синтезом употребом различитих сојева организама. У овом раду је клониран ген ChiA из *Bacillus licheniformis* DSM8785 који кодира хитиназу А (ChiA) са C-терминалним хексахистидином, а експримиран је у ванћелијском експресионом систему pYES2 из *Saccharomyces cerevisiae* INVSc1 као хипергликозилован ензим. Производња рекомбинантног ензима ChiA је са успехом потврђена тачкастим блотом употребом анти-His антитела. Утврђено је да је оптимално време експресије 24 h када је додата галактоза само на почетку ферментације, односно да након тог времена активност хитиназе опада. У другом експерименту је утврђено да се експресија наставља 72 h, уколико се галактоза додаје свака 24 h. Пречишћен ензим је детектован применом SDS-PAGE као хетерогена дифузна трака између 80 и 180 kDa. Упоређивана је молекулска маса ензима ChiA експримираног у *Pichia pastoris* KM71H, *Escherichia coli* BL21 (DE3) и *Saccharomyces cerevisiae* INVSc1 методом SDS-PAGE. Активност ензима ChiA је утврђена употребом флуорогеног супстрата 4-метилумбелиферил β-D-*N*,*N*,*N*-триацетилхитотриозида (4MUTC). Користећи биоинформатичку симулацију, постављена је хипотеза о могућем разлогу унутарћелијске експресије ензима. Претпоставка је да су број места гликозиловања кодираних у ChiA гену и близина ових места секвенци алфа фактора могући разлог овакве експресије.

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