Dextranase Expression in two Different Host-vector Systems of the Methylotrophic Yeast Pichia pastoris

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ABSTRACT

The expression of *dexA* gene from *Penicillium minioluteum* encoding a dextranase in two different strains of the methylotrophic yeast *Pichia pastoris* has been compared using vectors with two different auxotrophic markers. In both cases the *dexA* gene was fused to the *SUC2* signal peptide coding region from *Saccharomyces cerevisiae* and inserted in the genome of both strains, replacing the *AOX1* gene. Transformants specifying dextranase activity were identified as colonies surrounded by zones of clearing on blue dextran methanol plates. When using the MP36 strain *(his3⁻)* all selected transformants carried the expression cassette and secreted an active enzyme to the medium after methanol induction. However, in the case of the GS115 strain (*his4⁻*), only 50% of transformants expressed the active enzyme. We also found that the transformants in MP36 expressed higher levels of the enzyme than those in GS115, probably due to differences at the transcriptional level.

Keywords: AOX1 promoter, dextranase, heterologous gene expression, *Pichia pastoris*

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RESUMEN

Expresión de la enzima dextranasa en dos sistemas hospedero-vector de la levadura metilotrófica *Pichia pastoris*. Se comparó la expresión del gen *dexA* aislado del hongo *Penicillium minioluteum* y que codifica la enzima dextranasa, en dos cepas diferentes de la levadura metilotrófica *Pichia pastoris*, mediante el empleo de vectores que portan marcadores auxotróficos diferentes. En ambos casos, el gen *dexA* se fusionó a la secuencia señal del gen *SUC2* de *Saccharomyces cerevisiae* y se insertó en el genoma de ambas levaduras en el locus *AOX1*. Los transformantes capaces de secretar la enzima activa, fueron identificados por la aparición de zonas de aclaramiento alrededor de las colonias crecidas en placas que contienen dextrana azul y metanol como inductor. En el caso de la cepa MP36 *(his3⁻),* todos los transformantes seleccionados portaron el fragmento de expresión y secretaron dextranasa activa al medio de cultivo. Sin embargo, en el caso de la cepa GS115 *(his4⁻),* sólo 50% de los transformantes expresó la enzima activa. Además, los transformantes derivados de la cepa MP36 expresaron la enzima a niveles más altos que los derivados de la cepa GS115, probablemente debido a diferencias a nivel trasncripcional.

Palabras claves: dextranasa, expresión génica heteróloga, *Pichia pastoris,* promotor *AOX1*

*I*ntroduction

Saccharomyces cerevisiae is one of the best characterized yeasts for the production of heterologous proteins in eukaryotic organisms [1]. However, in recent years, alternative yeast species have gained popularity for this purpose. Among them, *Pichia pastoris* has become one of the most exploited for academic and commercial purposes [2]. This yeast offers several advantages, such as extremely high yields of intracellular proteins, very high levels of secretion into an almost protein-free medium; ease of fermentation to high cell density, genetic stability and possibilities of scaling-up without yield loss. In *P. pastoris* foreign genes are typically expressed under the control of the promoter of the yeast alcohol oxidase 1 gene *(AOX1)*. This promoter is fully repressed during growth of the yeast in glucose or glycerol and maximally induced during growth on methanol [3]. This tight regulation is useful in expression of foreign genes whose products may be toxic to the cell. The recent availability of this system as a kit (commercialized by Invitrogen Corp.) has allowed a wide use of this yeast as an expression system in the laboratory [4]. Our group have isolated a histidine auxotrophic mutant of *P. pastoris* affected in the gene encoding imidazol glycerol-P dehydratase (*HIS3*). This mutant, named MP36, could be successfully transformed with the *HIS3* gene from *S. cerevisiae* [5] and used as host for the expression of different heterologous proteins [6–11]. We decided to compare the effectiveness of this strain with that of the strain used by Invitrogen Corp. We compared the production of a fungal dextranase expressed in both strains with a vector carrying either *HIS3* or *HIS4* markers.

*M*aterials and Methods

Strains, plasmids and media

The yeast, bacterial strains and plasmids used in this work are shown in Table 1. The bacterial *Escherichia coli* strain MC 1061 used for plasmid propagation was grown in LB medium (1% triptona, 0.5% yeast extract, 0.2 M NaCl) with 50 µg/mL ampicillin. For selection and growth of yeast transformants, YNB minimal medium (Difco Laboratories, USA) supplemented with 2% of glucose was used.

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Table 1. Strains and plasmids used in this study.

Strains and plasmids	Genotype or phenotype	Reference
Strains		
E. coli MC 1061	*HsdR mcrB araD139 D(araABC-leu)7679 DlacX74 galU galK rpsL thi his3*	12
P. pastoris MP36	*his3* mutant	5
P. pastoris GS115	*his4* mutant	13
Plasmids		
pPDEX1	*dexA* gene in pPS7 plasmid	11
pPIC3K	Ap^r *HIS4*	14
pPICDEX	*dex* gene in pPIC3K plasmid	This study

Recombinant DNA methods

Cloning, DNA manipulations, and *E. coli* transformation were performed by standard techniques [15]. High specific activity labelling of hybridization probes was carried out by random hexamer priming [16] using [α-^{32}P]dATP (> 3000 Ci/mmol, Amersham, UK). For Southern blot analysis [17], DNA was transferred to Hybond-N membranes (Amersham, UK), and treated according to Sambrook *et al*. [15].

Ten micrograms of total RNA extracted from yeast cells were loaded onto 1% agarose/formaldehyde gels following standard protocols [15], and hybridization was carried out as described by Sambrook *et al*. [15]. The amount of RNA loaded was normalyzed by etidium bromide staining. DNA sequence analysis was performed by the dideoxy chain termination method of Sanger *et al*. [18]. Genomic DNA from yeast cells was prepared as described by Rothstein [19].

Construction of the *P. pastoris* expression vectors

Construction of pPDEX1 plasmid (Figure 1) has been previously described [11]. To express the dextranase enzyme into strain GS115 of *P. pastoris*, the dextranase-encoding region was amplified by PCR using the plasmid pPDEX1 as template [11]. The primers used in the reaction were 2738 (5'-TTGGATCCA TAATGCT TTTGCAAGCTTTCC-3') and 3079 (5'-GACTGATT ACCGTACACATATCGTCC-3'). The first oligonucleotide contains a *Bam*HI restriction site and hybridized to the 5' region of the *SUC2* signal peptide sequence. Primer 3079 hybridized to the 3' of the dextranase encoding region. The PCR product corresponding to dextranase gene plus the *SUC2* signal peptide coding region was digested with *Bam*HI and *Eco*RI, and the resulting 2 kb fragment was ligated into pPIC3K [14], which was previously digested with the same enzymes. In the resulting plasmid, named pPICDEX, the PCR product was under the control of the *AOX1* promoter and this fusion was checked up by sequencing using the *AOX1* 5' primer (5'- GACTGGTTCCAATTGACAAGC-3').

Transformation of *P. pastoris* and selection of dextranase-producing clones

About 10 µg of pPDEX1 and pPICDEX1 plasmid DNA were digested with *Pvu*II and *Bgl*II, respectively.

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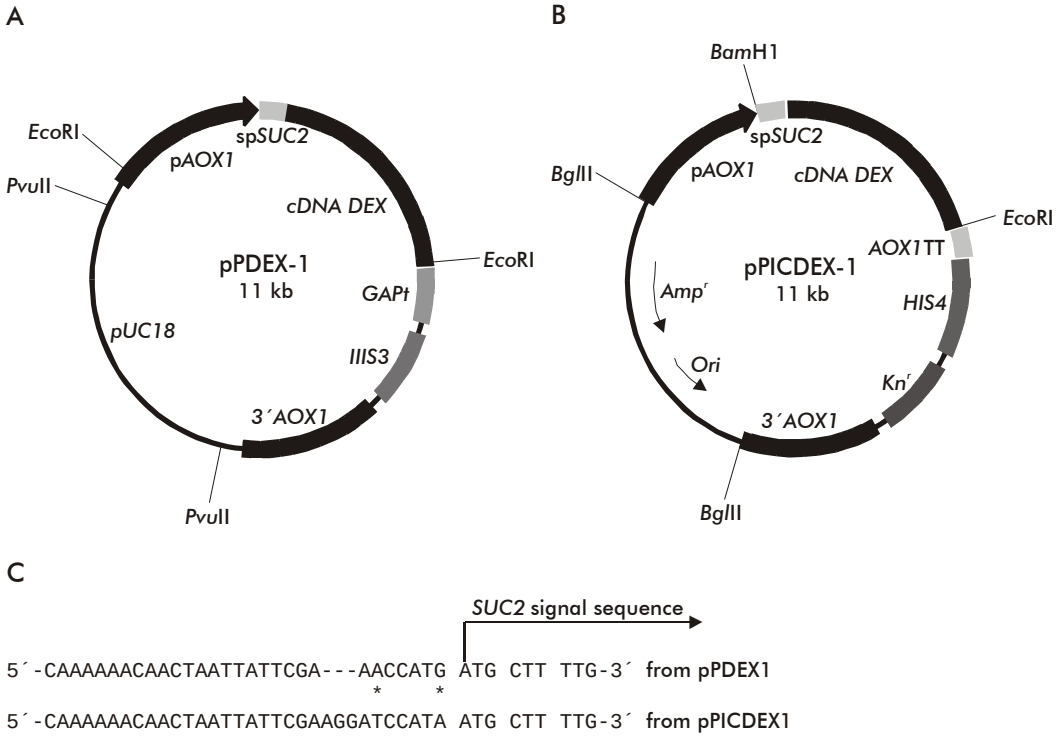

Figure 1. *P. pastoris* expression vectors used in this work. A) Vector pPDEX1 comprises a dextranase encoding gene under *AOX1* promoter (p*AOX1*), the signal sequence of *SUC2* gene (sp*SUC2*), *GAP* terminator (*GAPt*), *HIS3* gene from *S. cerevisiae,* and *AOX1* gene 3' region. B) Vector pPICDEX1 comprises *HIS4* gene from *P. pastoris* and the transcriptional terminator of *AOX1* gene (*AOX1*TT), instead of *HIS3* and *GAPt* from *S. cerevisiae,* respectively. Kn^r is the kanamycine-resistance marker for G418 selection of multicopy transformants. C) Alignment of the *AOX1* promoter 3' region fused to the *SUC2* signal sequence. The asterisk indicates nucleotide differences between two sequences and gaps have been introduced into the sequences of *AOX1* promoter from pPDEX1 plasmid to maximize similarities.

The digested plasmids were used to transform *P. pastoris* host strains MP36 and GS115 by electroporation. This procedure was carried out according to Becker and Guarente [20] with cells pulsed in 0.2 cm electroporation cuvettes at 1500 V, 25 mF, 200 W, using a BioRad Gene Pulser with Pulse Controller (BioRad, USA). One milliliter of cold 1 M sorbitol was added to the cuvettes immediately after pulsing, and His^+ transformants were recovered on minimal-agar plates [21] with 2% dextrose. About 100 colonies from each strain were transferred to minimal-agar plates with 0.4% blue dextran (Pharmacia), and dextranase expression was induced with methanol in vapor phase by addition of 0.2 mL portions of 100% methanol under the lids of inverted plates. After overnight incubation at 30 ºC the formation of clear halos of hydrolysis around the colonies were observed.

Shake-flask dextranase expression studies

Five positive transformants from both MP36 and GS115 strains were selected for dextranase expression studies in shake flasks. The cultures were grown in 50 mL of 0.67% YNB medium with 2% glycerol for 22 h at 30 ºC and 250 rpm using an incubator shaker (New Brunswick Scientific Co., USA). The cells were then centrifuged, washed twice with water and resuspended in the same medium with 1% methanol instead of glycerol, to induce the *AOX1* promoter and dextranase expression. After 24 h of growth in this medium, additional 0.5% methanol pulses were supplied every 12 h for 120 h. At this time the final dextranase activity in the culture supernatant was measured. The optical density at 530 nm (OD_{530}) of the culture samples were determined every 12 h. Samples taken at 0 and 42 h of methanol induction were used for RNA isolation.

Enzymatic assays

Dextranase activity was determined according to Kosaric *et al*. [22]. The reducing sugars formed were determined colorimetrically by the dinitrosalicylic acid reagent method [23]. One unit (1 U) is defined as the amount of enzyme that releases 1 mmol of glucose equivalents in 1 min from dextran T-2000 (Pharmacia, Sweden) at 40 ºC and pH 5.5.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [24]. Gels (12.5% acrylamide) were put in a Mighty Small II SE 250 electrophoresis system (Hoefer Scientific Instruments, San Francisco, USA), for protein separation.

Results and Discussion

Transformation of *P. pastoris* and selection of dextranase-producing clones

To compare effectiveness of different recipients and vectors for heterologous protein production in *P. pastoris*, the expression levels of *P. minioluteum* dextranase in strains MP36 and GS115, were measured respectively. The former strain is defective in the *his3* gene and can be transformed using plasmids with the *HIS3* gene from *S. cerevisiae;* the latter, which is defective in the *his4* gene, is transformed using the homologous *HIS4* gene from *P. pastoris*. To express the dextranase gene in the GS115 strain, the PCR fragment encoding the mature dextranase protein was subcloned into pPIC3K vector [14] to yield the expression plasmid pPICDEX1. This plasmid shares some characteristics in common with pPDEX1 plasmid [11], such as having the *dexA* gene under the control of the *AOX1* promoter and the use of *SUC2* signal peptide coding region from *S. cerevisiae* to direct the secretion of the enzyme. The *S. cerevisiae GAP* terminator works as efficiently as the *AOX1* terminator in *P. pastoris*, and the Kn^r gene present in the pPICDEX1 plasmid does not act as an autonomous replication sequence in this yeast. Therefore, the main difference between the two plasmids is the selection marker used (Figure 1). The pPDEX1 and pPICDEX1 plasmids were digested with *Pvu*II and *Bgl*II, to generate DNA fragments that were targeted to transplace into the *AOX1* locus of MP36 and GS115 strains, respectively. Transformation by electroporation of both strains yielded about 30 HIS^+ transformants/µg of DNA. All tested positive transformants (about 80) from MP36 strain secreted an active enzyme. However, in the case of GS115 strain, only 50% of the analyzed transformants formed halos of dextran hydrolysis (Figure 2).

The colonies that did not secrete an active enzyme could arise from gene conversion events, which repair the mutation at the *his4* locus without integration of the expression cassette into the yeast genome. This event was likely less frequent in MP36 strain due to the use of the *HIS3* gene from *S. cerevisiae.* This gene product has only a 67% of identity with the *HIS3* gene product from *P. pastoris* [25], which would reduce the frequency of gene conversion.

Analysis of the integration events

To prove the aforementioned hypothesis, which explains the results showed in Figure 2, chromosomal DNA was isolated from 12 transformants of each strain and analyzed by Southern blot. The DNAs were

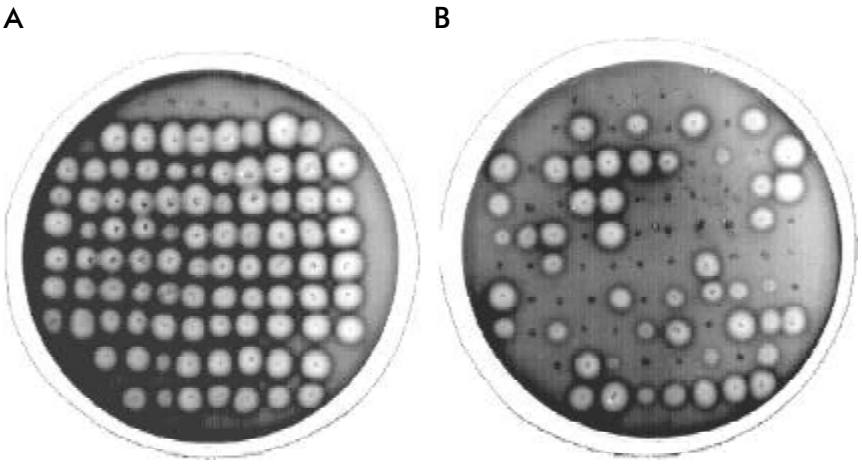

Figure 2. Halos formed in a bluedextran agar plate by transformants of *P. pastoris* secreting dextranase enzyme. A) MP36 strain transformed with pPDEX1. B) GS115 strain transformed with pPICDEX1 plasmid. Colonies on the upper part of the plate that did not form halos of dextran hydrolysis correspond to the untransformed GS115 and MP36 strains, respectively. Colonies at the bottom correspond to the previous clones of MP36 transformed with pPDEX1 [11]. This experiment was performed three times and the same result was obtained.

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digested with *Eco*RI, and hybridized to a dextranase probe. In MP36-derived transformants, the expression cassette was integrated in all of them, showing a single band of about 3.7 kb (Figure 3A). This pattern appeared in a single-copy transplacement clone and produced a Mut^s (slow methanol utilization) phenotype, meaning that the integration of the expression cassette into the chromosome of the MP36 host disrupted the *AOX1* locus.

In vivo circularization of the DNA fragment targeted for transplacement may occur in *Pichia* cells. Circularization of the *Pvu*II transplacement cassette from the pPDEX1 could allow its insertion into *AOX1* without disruption of the gene, since this can occur by single cross-over. Alternatively, circularized fragments could insert themselves wherever they are homologous to the chromosomal DNA; for instance at the *AOX2* (due to the fragment of the coding region of the *AOX1* gene included in the integrating plasmid) or *HIS3* loci. Examples of this type of clones can be seen in lanes 3, 6 and 9 of Figure 3A. This type of integration has been shown and analyzed previously by Clare *et al.* [26].

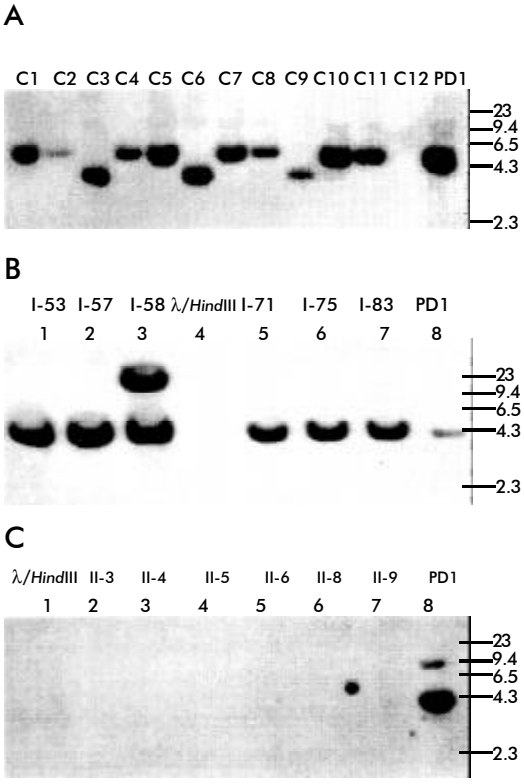

Figure 3. Southern blot analysis of chromosomal DNA from different *Pichia pastoris* integrative clones. Ten micrograms of genomic DNA were digested with *Eco*RI and the membrane was hybridized with a *dexA* specific probe (PCR product contained a dextranase gene). The corresponding DNA of different clones is indicated in each lane. DNA from PD1 strain [10] was used as control of a single-copy transplacement clone. A) Clones from MP36 strain. B) Clones from GS115 strains that formed halos of dextran hydrolysis. C) Clones from GS115 that did not express dextranase-encoding gene. Lanes 1, 2, 4, 5, 7, 8, 10 and 11 from panel A, and 1, 2, 5, 6 and 7 from panel B are single-copy transplacements clones with Mut^s phenotype. The molecular weight marker is indicated at the right of each Figure.

We analyzed six transformants from GS115 strain that formed halos in plates containing blue dextran, and six that were not halo producers. In DNA extracted from transformants that expressed an active enzyme, a single band of 3.7 kb was observed. As we mentioned above, this pattern corresponds to a single-copy transplacement and a Mut^s phenotype is obtained (Figure 3B). Southern blot analysis of chromosomal DNA from non expressing transformants showed the absence of the expression cassette in the genome of this type of clones (Figure 3C). The same membrane was hybridized using the *AOX1* promoter fragment as probe, and the expected 5.5 kb band was observed (data not shown). These results indicate that the lack of activity in several transformants derived from GS115 strain is due to the absence of the expression cassette in these cells.

The results obtained in the Southern blot analysis of several transformants from both strains showed that the majority of these (75% in MP36 transformants and 83% in GS115 transformants) are Mut^s, containing a single copy of the transplacement cassette.

Expression of *dexA* gene in transformants of both *P. pastoris* strains in shake flasks

Five well-characterized transformants that formed halos of dextran hydrolysis and with the same Mut^s phenotype (single-copy transplacement of *AOX1* locus), were selected. Mut^s clones grew more efficiently than Mut$^+$ in these conditions, possibly because of the fact that a Mut$^+$ strain is more likely to become oxygen-limited.

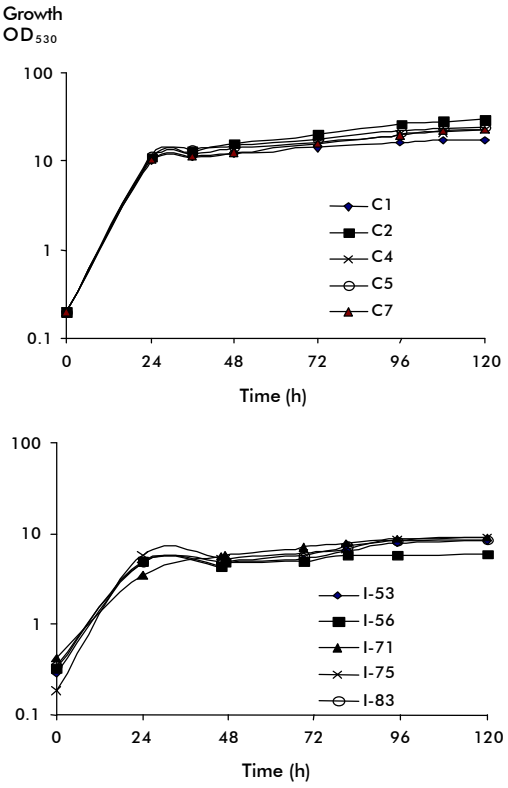

Figure 4. Optical densities of five transformants from MP36 (A) and GS115 (B) strains determined at 530 nm.

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The final optical density of the cultures of the GS115 transformants was half the values for the cultures of MP36 clones (Figure 4). In the case of MP36, the levels of dextranase activity varied between 38 U/ ML and 98 U/mL (Table 2). However, the enzymatic activity in GS115 transformants was three order of magnitude lower, even after buffering the medium to pH 6 (data not shown). In these strains the activity was about 0.04 U/mL (Table 2).

Samples of the culture supernatants from both recombinant strains were analyzed by SDS-PAGE. In the case of the MP36 transformants (Figure 5A), a strong protein band with a molecular mass corresponding to the native enzyme from *P. minioluteum* (lane 6), was observed. On the contrary, in the case of GS115 transformants (Figure 5B), only a weak band could be seen at the same molecular size after a 20-fold concentration of the samples. This result correlates with the low enzymatic activity obtained in the transformants from GS115 strain.

To seek a possible explanation for this result, Northern blot analyses were carried out. RNA isolated at 0 and 42 h after methanol induction from C2 and I-75 clones, was used. The hybridization signal obtained for the RNA isolated from C2 transformants was stronger than that of I-75 clone, indicating that there are more specific dextranase transcripts in the first case (Figure 6). This suggests that the differences in dextranase expression between these two systems may be due to differences in the steady state of the corresponding mRNA. Assuming a similar half-life for the mRNA in both strains, the most plausible explanation would be a difference in the level of transcription (Figure 6).

A high density fermentation of C2 and I-75 clones was performed and at the end of the run (120 h), the dextranase activity in the culture supernatant of MP36-derived transformat C2 was 980 U/mL, showing over a 10-fold increase in respect to the shake-flask experiments. In the case of GS115 transformants (I-75 clone), we observed a 1000-fold increase in yield, when switching from shake flasks to fermenter. However, dextranase activity remained extremely low about 60 U/mL.

Table 2. Dextranase induction in 50 mL shake-flask cultures of five transformants derived from MP36 and GS115 strains. The level of secreted dextranase was quantified by measuring the enzymatic activity in the culture supernatant.

MP36 transformants			GS115 transformants		
Clones	OD_{530}	Dextranase activity μmoles/min/mL (U/mL)	Clones	OD_{530}	Dextranase activity μmoles/min/mL (U/mL)
C1	18	38	1-53	8	0.05
C2	30	98	1-56	6	0.04
C4	23	74	1-71	9	0.05
C5	23	67	1-75	9	0.05
C7	25	62	1-83	8	0.03

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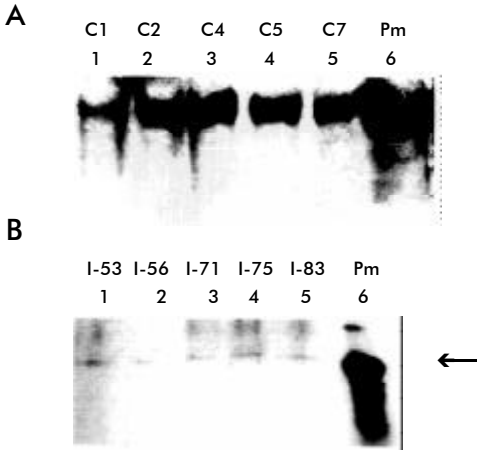

Figure 5. SDS-PAGE analysis in 12.5% gels of the recombinant dextranase expressed in *P. pastoris*. A) Culture supernatant (7 μL) from five MP36 transformants (lanes 1 to 5). B) Culture supernatant (7 μL) from five GS115 transformants (lanes 1 to 5). In the latter case, the culture supernatant (50 mL) was previously concentrated 20-fold. Lane 6 in both panels corresponds to the native *P. minioluteum* enzyme (Pm). The arrow indicates the position of dextranase protein (67 kDa).

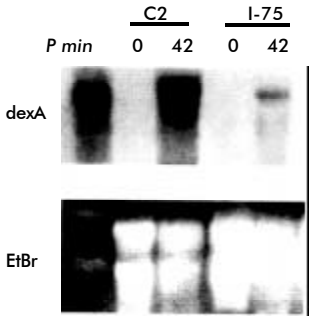

Figure 6. Northern blot analysis of RNA isolated at 0 and 42 h after methanol induction of C2 and I-75 transformants derived from MP36 and GS115 strains, respectively. RNA isolated from the fungus *Penicillium minioluteum (P min)* grown in dextran-containing medium, was used as positive control. The hybridization was performed with the dextranase gene as probe *(dexA)*. Samples from *P min* and C2 strains were incubated overnight at -70 °C and samples from I-75 strain were incubated in the same conditions for 96 h. EtBr represents an ethidium bromide-stained gel.

Our results clearly show differences between these two expression systems in the methylotrophic yeast *P. pastoris*. The most remarkable difference was that MP36 transformants were more efficient in the expression of dextranase gene than GS115 strains. One possible explanation could be that there are differences between the genetic background of these two strains.

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