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EXPERIMENTAL ARTICLES

Expression of Inulinase Genes in the Yeasts *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*

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Abstract—Expression of the genes encoding the enzymes involved in inulin, sucrose, and glucose metabolism in the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* was studied. The exon–intron structure of the relevant genes was identified and the primers for quantitative PCR were optimized. Expression of the genes was found to depend on the carbon source. Glucose was shown to exhibit a repressive effect on inulinase synthesis by *K. marxianus*, while sucrose induced it, and in *S. cerevisiae* glucose and sucrose were inulinase inducer and repressor, respectively.

Keywords: Saccharomyces cerevisiae, Kluyveromyces marxianus, carbon source, glycosidase, inulinase, expression, PCR, primer

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Glycoside hydrolases, or glycosidases (EC 3.2.1), represent one of the best-studied and the most commonly applied group of enzymes. It includes the enzymes of the GH32 family, which hydrolyze the glycoside bonds of carbohydrates, e.g., sucrose, various oligo- and polysaccharides, and fructans (levan and inulin). Inulinase (2,l-β-D-fructan fructanohydrolase, EC 3.2.1.7) is an enzyme of a considerable practical importance, since it hydrolyzes β-2,1 bonds of inulin and produces fructose and fructo-oligosaccharides widely applied in medicine and the food industry to obtain functional nutrition products. The use of inulinase-mediated hydrolysis of inulin-containing material enables single-stage enzymatic production of fructose eliminating the need for using α-amylase, glucoamylase, and glucose isomerase, which are involved in fructose production from starch. Inulinases are produced by 15 fungal genera, 12 yeast genera, and 8 bacterial genera. Yeasts are better inulinase producers than fungi and bacteria, are more easily cultured, and synthesize larger amounts of the enzyme [1, 2]. Among them, *Kluyveromyces marxianus* is the best-studied and the most widely applied inulinase-producing species. Currently, *K. marxianus* strains with inulinase activity are used for production of ethanol biofuel from inulin-containing material in a process that combines the saccharification and fermentation stages [3]. At the same time, there are few known inulinase producers among *Saccharomyces cerevisiae* strains; the enzyme properties and the mechanisms of its regulation remain insufficiently studied [4, 5]. For the purposes of wider inulinase application, it is necessary to identify new enzyme sources, investigate its properties, and well as the structure and regulation of the relevant genes.

Most studies concerning the regulation of inulinase gene expression in yeasts have been performed on different *K. marxianus* strains. Some data suggest that inulinase production depends on the carbon source. Gupta et al. showed that, in *K. marxianus*, glucose caused catabolic repression of inulinase production, whereas sucrose and fructose acted as inulinase inducers, although weaker than inulin [6]. At the same time, no inulinase induction mechanisms were identified in another *K. marxianus* strain, CDBB-L-278 [7]. In a study by Gao et al., inulinase activity in *K. marxianus* YX01 was higher when the carbon source employed was inulin, rather than glucose or fructose; glucose had a repressing effect on inulinase activity and in higher concentrations acted as an inhibitor [3].

Investigation of the effects of different carbohydrates on inulinase biosynthesis in *S. cerevisiae* VPSh-2 showed that the enzyme production could be induced by fructose, sucrose, and inulin. In the glucose-containing medium, inulinase activity was relatively low [4]. In a work by Silva-Santisteban et al. it was concluded that inulinase synthesis was regulated in a complex manner that depended on the strain [8]. The currently available data are insufficient to propose a detailed model describing the regulation of inulinase synthesis. Therefore, comparative analysis of the regulation of β-fructosidase gene expression in the yeasts *K. marxianus* and *S. cerevisiae* can provide important information.

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The goal of the present work was to investigate the regulation of the inulinase gene expression in the yeasts *S. cerevisiae* and *K. marxianus* growing in media with different carbon sources (inulin, sucrose, or glucose) using quantitative RT–PCR.

MATERIALS AND METHODS

The study was performed with yeast strains *K. marxianus* Y-1148 (All-Russian Collection of Microorganisms (VKM), Institute of Biochemistry and Physiology of Microorganisms, Russia) and *S. cerevisiae* G, derived from *S. cerevisiae* strain Y-2902D (VKM) [9].

Yeasts were grown in a synthetic medium containing the following (g/L tap water): carbon source (sucrose, glucose, or inulin), 20.0; $(NH_4)_2SO_4$, 5.0; KH_2PO_4, 0.85; K_2HPO_4, 0.15; $MgSO_4$, 0.5; NaCl 0.1; $CaCl_2$ 0.1; yeast extract, 2.0; pH 5.0. Inulin was obtained from Beneo^{tm}GR (Orafti®HR, Belgium). The medium was sterilized for 20 min at 121°C and inoculated to the concentration of 10^7 cells/mL medium with yeast suspension obtained by washout from wort agar. Yeasts were grown at 30°C for 24 h on a temperature-controlled shaker at 200 rpm in 250-mL flasks containing 50 mL medium. Yeast biomass was collected by centrifugation at 2600 *g* for 10 min at 2°C and used to obtain water extracts of intracellular inulinase, while the supernatant was used to determine the extracellular enzyme contained in the culture liquid. To isolate the intracellular enzyme, the biomass was washed with water and precipitated by centrifugation, then dried to 75% humidity and ground in a mortar with glass sand and distilled water in a 1 : 10 ratio. The ground biomass was incubated at 20°C for 20 min and then centrifuged to remove cell debris and sand; the supernatant was used to determine the intracellular inulinase activity.

Inulinase activity was determined by quantifying the reductive agents released in the course of the enzyme-driven substrate hydrolysis. The quantity of enzyme that catalyzed the production of 1 μg of reductive agents in 1 min under the experimental conditions was accepted as the inulinase activity unit. To determine inulinase activity, 2 cm^3 of 5% (wt/wt) inulin solution in 0.1 mol/dm^3 acetate buffer (pH 4.7) were mixed with 1 cm^3 of yeast extract; the mixture was incubated at 50°C for 20 min. The control sample was mixed with an enzyme extract that has been heated in a boiling water bath for 1 to 2 min. Fructose content in the specimens was determined using the method proposed by Pochinok as described in [10]. Inulinase activity was calculated using the following formula:

$$A = \frac{A_0 - A_n}{180n\tau},$$

where A is the enzyme activity, U/g; A_0 is the amount of reductive compounds produced by inulin hydrolysis, μg; A_n is the amount of reductive compounds in the control sample, μg; n is the enzyme extract amount, cm^3; and τ is the time of hydrolysis, min.

To investigate the regulation of expression of the inulinase gene in different yeast strains, primers were designed to β-fructosidase genes of *S. cerevisiae* and *K. marxianus*. The primers were designed based on exon sequences of β-fructosidase genes of *K. marxianus* and *S. cerevisiae* from the NCBI database [11]. Homologous sequence fragments were identified by multiple alignment of gene sequences using the AliBee online service [12]. The primer annealing temperatures and the probabilities of hairpin formation and cross-linking were determined using the FastPCR software package.

Nucleic acid specimens were isolated and purified according to the standard protocol described in [13]. The obtained RNA was used to synthesize cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Sileks, cat. no. E1211) with oligo-(dT) primers as recommended by the manufacturer.

The obtained DNA was used in quantitative PCR performed in the following reaction mixture: 2.5 μL 10× PCR buffer (100 mM Tris–HCl, pH 8.8; 500 mM KCl), 25 mM $MgCl_2$, 10 mM dNTP mixture, 10 mM primers, 1 U *Taq* DNA polymerase, 0.1 μL SYBR Green dye (1 : 10000 dilution), and 1 μL cDNA (20 ng).

Amplification was performed in a CFX96 thermal cycler (Touch Real Time System, Bio-Rad, United States) according to the following protocol: preliminary denaturation at 95°C for 5 min; 35 cycles of 40 s denaturation at 95°C, 40 s annealing at 60°C, and 60 s elongation at 72°C; detection of products' fluorescence; and final elongation for 5 min at 72°C; cooling of the reaction mixture.

The results of quantitative PCR were normalized to the data obtained for four housekeeping genes: *ALG9, TAF10, TFC1*, and *UBC6* [14, 15].

Amplified DNA fragments were separated by electrophoresis in a 1% agarose gel.

RESULTS AND DISCUSSION

The highest levels of inulinase activity were observed in the yeasts grown in inulin-containing medium; i.e., inulin acted as an inducer. The activity of the *S. cerevisiae* G enzyme was nearly four times higher than of the *K. marxianus* Y-1148 enzyme (table). The inulinase activity in *K. marxianus* Y-1148 grown in sucrose medium was 1.5-fold higher than in the case of glucose medium, while in the latter it was two times lower than in the inulin medium. Thus, in this strain, inulinase production was inducible and subject to glucose-mediated catabolic repression. In *S. cerevisiae* G grown in the glucose medium, inulinase activity was two times higher than in the case of

sucrose medium and nearly two times lower than in the case of inulin medium; that is it was sucrose that had a repressing effect.

To investigate the expression of the inulinase genes in *K. marxianus* Y-1148 and *S. cerevisiae* G, primers were designed to the genes encoding enzymes of the β-fructosidase class in *S. cerevisiae* and *K. marxianus*. The primers were designed using gene sequences encoding several *K. marxianus* and *S. cerevisiae* enzymes (endo- and exoinulinases, β-fructofuranosidases). It was found that all these genes had approximately the same size (2983 to 3325 bp) and contained one exon 1436 to 1669 bp long. Sequence alignment showed that all these genes had a fairly high degree of homology (65 to 97%). Homology between the *K. marxianus* inulinase gene and the *S. cerevisiae* invertase gene was 67%, whereas the endo- and exoinulinase genes of *K. marxianus* showed 97.35% similarity. These results suggest that all these genes and the enzymes in question share a common evolutionary origin.

The primers IPFYSK f 5'-GGKTTGTGGTACGATG-3' (forward) and IPFYSK r 5'-AGTGTTGAAGAAAGTTTGYA-3' (reverse) flank an exon fragment varying in length from 760 to 790 bp depending on the gene and the source object.

Total RNA specimens (0.5–0.8 ng) were isolated from yeast cells using the guanidine isothiocyanate technique, and their quality was verified by spectrophotometry and RNA electrophoresis in agarose gel. Reverse transcription with oligo-dT primers was used to synthesize cDNA from mRNA templates, and quantitative PCR was performed with IPFYSK primers. The presence of amplicons of the expected size of 760–790 bp was confirmed by electrophoresis (Fig. 1).

Analysis of normalized expression data showed that β-fructosidase genes were expressed in *S. cerevisiae* G and *K. marxianus* Y-1148 in all cultures growing in the media containing sucrose, glucose, or inulin (Fig. 2).

The expression levels of enzyme-encoding genes in *S. cerevisiae* G and *K. marxianus* Y-1148 depended on the carbon source present in the medium. In particular, in *S. cerevisiae*, it was the highest in the glucose-containing medium, whereas in the inulin-containing medium it was 1.6 times lower but still twice as a high as in the medium with sucrose. In *K. marxianus* Y-1148 growing on inulin and on sucrose, the gene expression levels were similar and corresponded to those observed in *S. cerevisiae* G growing on inulin; in *K. marxianus* Y-1148 cultured in the glucose-containing medium, this level was 1.8 times lower.

Our experiments showed that, for *K. marxianus* Y-1148, sucrose acted as an inducer of gene expression, and glucose acted as a repressor; whereas for *S. cerevisiae* G, glucose acted as an inducer, and sucrose, as a repressor. Thus, depending on the composition of the culture medium, we observed the expression of different genes whose activation was required for enzyme synthesis and carbohydrate metabolism in the given medium.

Inulinase activity in the yeasts grown in the media containing different carbon sources

Carbon source	Inulinase activity, U/g biomass	
	K. marxianus Y-1148	*S. cerevisiae* G
Inulin	150	590
Glucose	78	334
Sucrose	120	173

To analyze the homogeneity of the nucleotide composition of the amplicons in question, we obtained the melting curves of amplification products. The melting curves of PCR products obtained for *S. cerevisiae* G and *K. marxianus* Y-1148 had three T_m peaks, one for each culture medium (Fig. 3).

Our data indicate that PCR products vary among growing yeasts by metabolizing different carbohydrates and that the yeast strains studied contain the genes responsible for synthesis of the enzymes involved in metabolism of glucose, sucrose, and inulin. Our results suggest that, depending on the carbon source present in the culture medium, *K. marxianus* Y-1148 and *S. cerevisiae* G cells express those genes

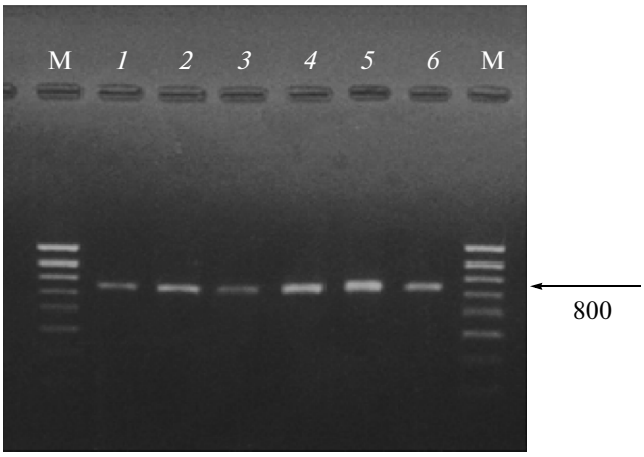

Fig. 1. PCR-amplified products β-fructosidase gene fragments of *S. cerevisiae* G and *K. marxianus* Y-1148 grown in the media containing a sole carbon source: glucose (*1, 4*), sucrose (*2, 5*), or inulin (*3, 6*): (*1–3*), *S. cerevisiae* G; (*4–6*), *K. marxianus* Y-1148; M, fragment length marker, 80 to 1031 bp (Lot 00078035).

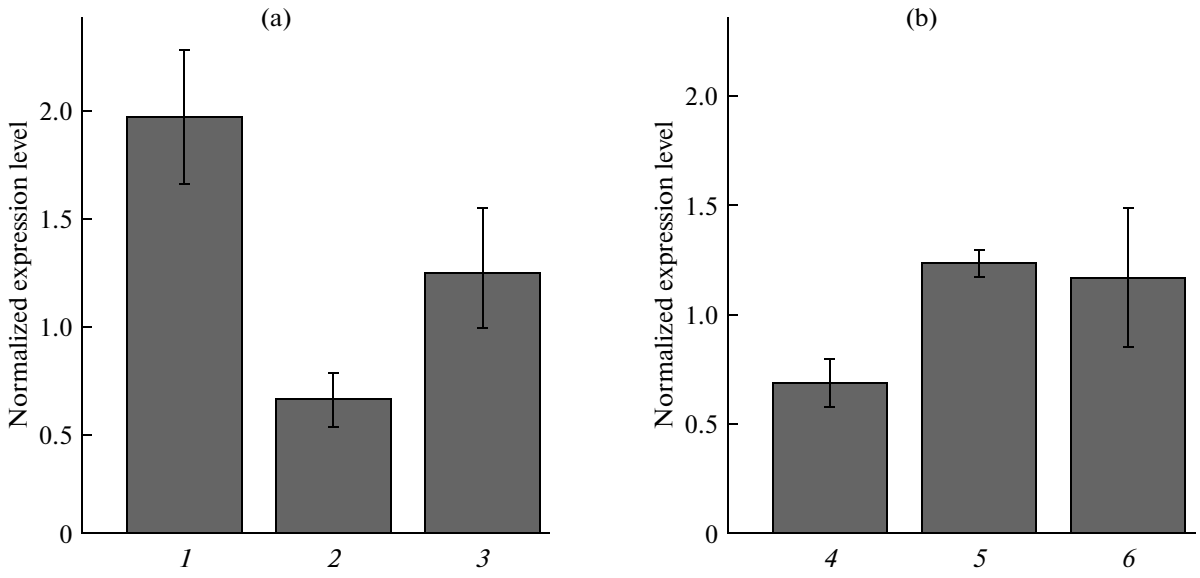

Fig. 2. Normalized expression levels of β-fructosidase genes in the yeasts grown in the media with a sole carbon source: (a) *S. cerevisiae* G on glucose (*1*), sucrose (*2*), and inulin (*3*); (b) *K. marxianus* Y-1148 on glucose (*4*), sucrose (*5*), and inulin (*6*).

that produce the enzymes metabolizing the particular carbohydrate. It was found that the expression levels of the genes involved in metabolism of carbohydrates (inulin, sucrose, or glucose) depended on the available carbon source. In our experiments, in *K. marxianus* Y-1148, glucose suppressed the expression of these genes, while sucrose stimulated them; at the same time, in *S. cerevisiae* G, glucose upregulated, and sucrose downregulated them.

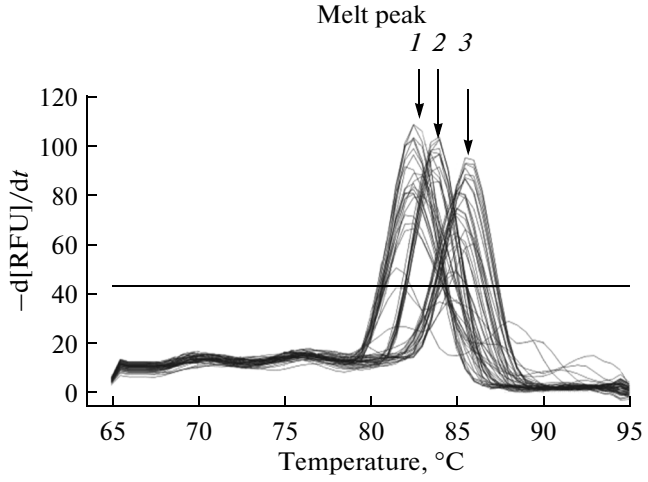

Fig. 3. T_m peaks and dissociation curves of obtained for *S. cerevisiae* G and *K. marxianus* Y-1148: (*1*), sucrose medium (T_m, 82.5°C); (*2*), inulin medium (T_m, 83.9°C); (*3*), glucose medium, (T_m, 86.0°C).

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