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Comparison of Promoters Suitable for Regulated Overexpression of β-Galactosidase in the Alkane-Utilizing Yeast *Yarrowia lipolytica*

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Abstract Promoters of the genes *G3P, ICL1, POT1, POX1, POX2* and *POX5* of the yeast *Y. lipolytica* were studied in respect to their regulations and activities during growth on different carbon sources. The aim of this study was to select suitable promoters for high expression of heterologous genes in this yeast. For this purpose the promoters were fused with the reporter gene *lacZ* of *E. coli* and integrated as single copies into the genome of *Y. lipolytica* strain PO1d. The measurement of expressed activities of β-galactosidase revealed that *pICL1, pPOX2* and *pPOT1* are the strongest regulable promoters available for *Y. lipolytica*, at present. *pPOX2* and *pPOT1* were highly induced during growth on oleic acid and were completely repressed by glucose and glycerol. *pICL1* was strongly inducible by ethanol besides alkanes and fatty acids, however, not completely repressible by glucose or glycerol. Ricinoleic acid methyl ester appeared as a very strong inducer for *pPOT1* and *pPOX2*, in spite of that it inhibited growth of *Y. lipolytica* transformants.

Keywords: *Yarrowia lipolytica*, expression vectors, regulated promoters, *lacZ* expression

INTRODUCTION

The group of the so called "non-conventional" yeasts, which addresses all other yeast species except *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, has received more and more attention in fundamental research and biotechnology in the last two decades. The ascomycetous, dimorphic yeast *Yarrowia lipolytica* is one of the few species among them which were more intensively studied in respect to its genetics, molecular biology and biotechnological applications [1,2]. This yeast is of special interest as a host for foreign gene expression and protein secretion because of its capability to secrete large proteins in high amounts [3-6]. Most of the genetic tools, like autonomously replicating plasmids [7-11] and gene amplification systems using multiple integrative plasmids [12,13] have been developed, as well as efficient secretion signals for export of proteins [14-16] have been characterized for *Y. lipolytica*. There are also a few promoters available which are suitable for regulated high expression of foreign genes in this yeast. The promoter of the *XPR2* gene (*pXPR2*) is the best studied and most often used among them [6,17-20]. The *XPR2* promoter is regulable by the pH value and the carbon or nitrogen source in the medium, however, it has the disadvantage that induction is possible only in rich, protein containing media, but not in minimal media. In the last years several new genes have been cloned and characterized from *Y. lipolytica* opening the chance to select a promoter for heterologous gene expression, which enables regulated high level of expression in minimal medium.

Studies on the activity of the glyoxylate cycle, the β-oxidation of fatty acids and the glycerol metabolism in *Y. lipolytica* have shown that there exist several highly expressed genes which are inducible/repressible by different carbon sources. Among them the genes encoding glycerol-3-phosphate dehydrogenase (*G3P*) (acc. no: AJ250328), isocitrate lyase (*ICL1*, acc. no: X72848) [21], 3-oxo-acyl-CoA thiolase (*POT1*, acc. no: X69988) [22], and acyl-CoA oxidases (*POX1, POX2, POX5*, acc. no.: AJ001299, AJ001300, AJ001303, respectively) [23,24] are well studied. It was, therefore, the aim of this investigation to compare the promoters of the genes *G3P, ICL1, POT1, POX1, POX2* and *POX5* among themselves and with the *XPR2* promoter in respect to their regulation by the carbon source of the medium and their strength of triggering gene expression. For this purpose these promoters were fused with the reporter gene *lacZ* from *E. coli* and integrated as single copies into the genome of a haploid strain of *Y. lipolytica* to compare expressed β-galactosidase activities.

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MATERIALS AND METHODS

Strains, Media, and Culture Conditions

Transformation and amplification of recombinant plasmid DNA was done in *E. coli* DH5αc (*supE44 ΔlacU169 (ϕ80 lacZ ΔM 15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*). *E. coli* cells were grown at 37°C in LB medium [25] or in LB medium containing ampicillin (100 μg/mL) in case of transformed cells.

Y. lipolytica strain PO1d (*MATA leu2-270 ura3-302 xpr2-322 SUC2*) [12] was used for expression studies. The *Y. lipolytica* strains were grown at 28°C in complete medium YPD [1] or in minimal medium yeast nitrogen base (YNB, Difco) with 1% of different carbon sources (glucose, glycerol, ethanol, oleic acid, ricinoleic acid methyl ester, dodecane, hexadecane) as indicated in the text. Oleic acid and ricinoleic acid methyl ester were given together with Tween 80 (final concentration 0.05%) as emulsifier and suspensions were sonicated before inoculation. Dodecane and hexadecane were supplied to a filter paper in the lid of the petri dishes as described [26].

Minimal medium was adjusted to pH 6.8 with 50 mM sodium/potassium phosphate buffer. Uracil and thiamine were supplied to minimal medium at 40 mg/L and 300 μg/L, respectively.

Integrative transformants of *Y. lipolytica* were selected on solid YNB with 2% glucose and uracil (YNB) [23,24]. Agar (20 g/L) was added for preparation of solid media.

For induction experiments, cells from a 24 h YPD culture were centrifuged, washed twice with minimal medium without carbon source and used to inoculate the culture in YNB with carbon source at an initial cell concentration of OD_{600} 0.5 to 1.

For β-galactosidase plate assay *Y. lipolytica* transformants on YNB with one of the carbon sources and 40 μg/mL X-gal together with of 50 mM phosphate buffer (pH 7.4) was used.

DNA Manipulations and Plasmids Constructions

All basic DNA manipulation procedures were performed according to Sambrook *et al.* [25] and Ausubel *et al.* [27]

The plasmids pINA-G3P, pIL43 [28], pINA-POX1, pINA-POX2, pINA-POX5 [24], pSXPr [29] were used for PCR based amplification or direct isolation of the promoter-*lacZ* fusions of the genes *G3P, ICL1, POT1, POX1, POX2* and *POX5*, respectively. Primer used for PCR based construction of links between the *lacZ* gene and the inserted promoters as well as primers used for creation of probes for Southern blot hybridization are shown in Table 1.

For construction of single-copy expression vectors the plasmid pINA354b, a derivative of plasmid pINA354 [17], was used (Fig. 1). This plasmid was constructed by deletion of the *Bam*HI site located downstream to the *lacZ* gene in pINA354. For this purpose, plasmid pINA354 was partially digested with *Bam*HI and filled in with T4 polymerase. Enzymes were heat inactivated and plasmid was religated before transformation of *E. coli* DH5αc. Deletion of the desired restriction site was tested by restriction analysis. pINA354b contains a hybrid promoter (XPR2c=hp4d) [20,30] fused with the 5′ end of the *lacZ* gene of *E. coli*, the terminator of the *XPR2* gene (*XPR2*ter) of *Y. lipolytica* at the 3′ end of *lacZ*, the *LEU2* gene as a marker for selection in *Y. lipolytica* and the amp^R gene for selection of transformants in *E. coli* (Fig. 1). Plasmid Bluescript KS^+ (Stratagene, La Jolla, CA, USA) was used for subcloning the PCR created DNA fragments containing the promoters of the *POX1, POX2* and *POX5* genes.

Table 1. List of primers used for PCR based construction of links between the *lacZ* gene and the inserted promoters in pINA354b derivatives and creation of probes for Southern hybridisation. Underlined sequences mark the restriction sites for *Bam*HI and *Not*I

Primer	Sequence in 5′ – 3′ direction
A174N1	gcc gca ctc gtg cgg ccg cag ctt cct atg cgc ctt tct ctc tgc
A174N2	gac gca aaa ttt gtg tac agg cgc cta ggg gca gag
A174N3	ggt ggc agc ggt tct aac agc gcc tag gga cgt gac gag tg
LacZ-17	aaa cgg cgg att gac cg
ICL-GAL	ccc gta cta acc cag tgc aag ctt gcg atg ccg tc
aco1-P1	act gtg cgg ccg ctc ggg gag ctt tct gc
aco1-P2	ctc gag tgg atc cag gtt gct tcc atg gcc caa c
aco2-P1	aca cag cgg ccg cac ata ctg tta cac tgc tcc g
aco2-P2	ctc gag agg atc cag gtt gga gcc atg gcc cag
aco5-P1	ggg ggc ggc cgcggt acc cat gac gta caa acc c
aco5-P2	ctc gag agg atc cac gtt aga tcc gtg acc cag

Transformation of *Y. lipolytica* and of *E. coli* Cells

Transformation of *Y. lipolytica* was performed by the lithium acetate method as previously described [1]. Transformation of *E. coli* was carried out according to Sambrook *et al.* [25].

Chromosomal DNA Preparation and Southern Blot Analysis

Chromosomal DNA of *Y. lipolytica* transformants was prepared according to Barth and Gaillardin [1], digested with *Apa*I and used for Southern blot analysis. The probe used for estimation of single copy integration was prepared by PCR from plasmid pINA354b using the primers lacZ-17 and ICL-GAL (Table 1). Labelling of this probe containing a C-terminal fragment of the *lacZ* gene (299 bp) and detection in Southern blot hybridization was performed using the random prime labelling and detection system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK).

DNA Sequencing

DNA sequencing was carried out according to Sanger

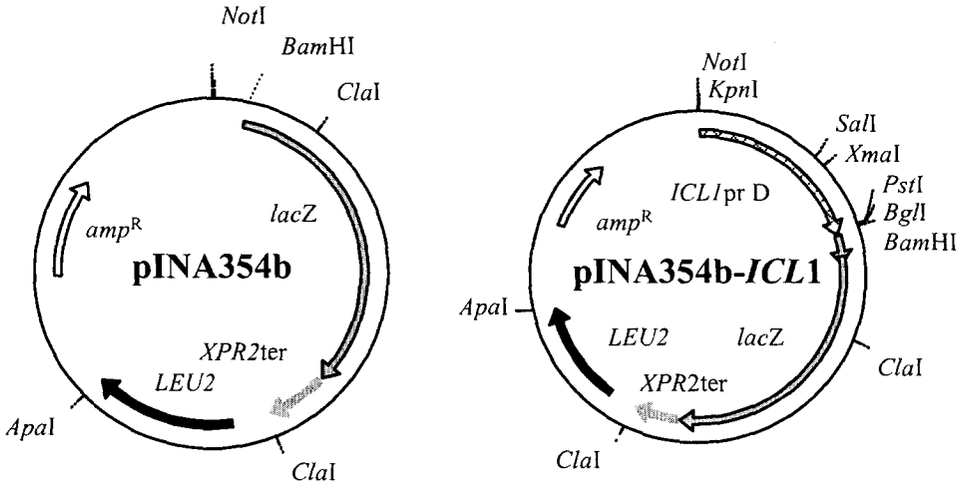

Fig. 1. Single copy integrative vectors for studying the regulation and expression strength of *Y. lipolytica* promoters. Plasmid pINA354b was used as basic vector for insertion of different promoters by exchanging the hybrid promoter hp4d in front of the *lacZ* gene. pINA354b-*ICL1* is shown as representative for the resulting other constructions used. The promoter-*lacZ* fusions were created by PCR and inserted in *Not*I and *Bam*HI. The derived plasmids were linearized by *Apa*I or *Xma*I and integrated into the *leu2*-270 or *ICL1* loci, respectively, in the *Y. lipolytica* strain PO1d. *ICL1*prD = promoter of the *ICL1* gene followed by the intron in the ATG of *ICL1*, *XPR2*ter = terminator of the *XPR2* gene, *lacZ* = β-galactosidase gene of *E. coli*, amp^R = ampicillin resistant gene.

et al. [31] using the multiwell sequencing kit of Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK).

Determination of β-Galactosidase Activity

The β-galactosidase activity was measured by the toluene permeabilized cell assay as described previously [27,32]. In each case double measurements of three independently performed cultivation of transformants were done.

RESULTS AND DISCUSSION

Vector Construction

We have chosen the genes *G3P*, *ICL1*, *POT1*, *POX1*, *POX2* and *POX5* to check their promoters for usage in heterologous gene expression in *Y. lipolytica*. For this purpose the promoters were introduced in pINA354b fused to the 5′ end of the *lacZ* gene of *E. coli*. The *Not*I/*Cla*I fragment from plasmid pIL43 [28] containing the *ICL1* promoter with part of the 5′ end of the *lacZ* gene was inserted into the *Not*I/*Cla*I (partially) digested vector pINA354b getting plasmid pINA354b-ICL1 (Fig. 1). The *ICL1* promoter contained still the intron of the *ICL1* gene, which is correctly spliced and has no effect on expression level [28].

Promoter of gene *POT1* was isolated as *Not*I/*Cla*I fragment from plasmid pSXPR and also inserted into the *Not*I/*Cla*I (partially) digested vector pINA354b resulting in plasmid pINA354b-POT1.

Construction of vectors for chromosomal integration of *POX1*, *POX2*, and *POX5* promoter-*lacZ* gene fusions were done in following way. The *POX1* promoter and part of the ORF was amplified from plasmid pINA-*POX1* DNA using the primers (Table 1): *aco1*-P1 (this being equivalent to region -1800 to -1772 of the *POX1* promoter and was used to generate a *Not*I site) and *aco*1-P2 (is complementary to the +400 to +430 of the *POX1* ORF and was used to generate a *Bam*HI site and corresponding to a translational fusion at amino acid 100). The amplified 1.89 kb fragment was cloned in Bluescript KS^+ and checked by DNA sequencing. The resulting plasmid KS-*POX1*p was then digested with *Not*I and partially with *Bam*HI and used to replace the *Not*I-*Bam*HI *XPR2* fragment of the integrative vector pINA354b. The resulting plasmid called pINA354b-*POX1*p was introduced by transformation into *Y. lipolytica* strain PO1d. The same procedure was done for the construction of pINA354b-*POX2*p and pINA354b-*POX5*p using primer combinations aco2-P1/aco2-P2 and aco5-P1/aco5-P2, respectively.

The *G3P* gene harbours at the 5′ end an intron. To test whether the intron in the *G3P* gene has any effect on expression level two fusions with *lacZ* were constructed. In first case *lacZ* was directly fused to the ATG of the ORF of *G3P* (G3PB1), in second case the ORF of *lacZ* started after the end of the intron in the *G3P* gene (G3PB2). These two sequences of the *G3P* promoter were synthesized by PCR from plasmid pINA-*G3P* using the primer combinations A174N1/A174N2 and A174N1/A174N3 (Table 1) and as *Not*I/

*Bam*HI fragments inserted into the vector pINA354b.

The recombinant plasmids were amplified in *E. coli* DH5αc and the sequences of the expression cassettes were confirmed by DNA sequencing. These recombinant plasmids were linearized with *Apa*I, except pINA354b-ICL1 which was digested with *Xma*I. Linearized plasmids were used for transformation of *Y. lipolytica* strain PO1d resulting in appearance of integrative transformants after 3-4 days cultivation on YNBcas plates. Additionally, a hybrid promoter (*pXPR2$_c$ = php4d*), which contains four copies of the UAS1B region of the *XPR2* promoter cloned upstream of the *LEU2* minimal TATA box resulting in constitutive expression of the controlled gene [30], was also fused with the *lacZ* gene and transformed into strain PO1d. A transformant containing this constitutive promoter and a transformant harbouring *lacZ* under control of the original *XPR2* promoter (plasmid pINA354) was included for comparison in the following studies.

One clone from each transformation was selected for further studies in a two step procedure. At first, clones were screened for their *lacZ* expression on X-gal medium with oleic acid as inducer of all promoters, except the *XPR2* promoter. In the second step the selected transformants were checked by Southern blot hybridization for the presence of only one integrated copy of plasmid DNA per cell (data not shown). *Apa*I cut plasmids were directed to the *LEU2* gene for integration, whereas the *Xma*I linearized vector pINA354b-ICL1 was integrated into the promoter of the chromosomal copy of the *ICL1* gene.

Expression of the *lacZ* Gene under Control of Different Promoters

To check which carbon source induces which promoter a preliminary plate test was performed. In this test each of the transformants described above was cultivated on X-gal containing minimal medium with either glucose, glycerol, ethanol, oleic acid, ricinoleic acid methyl ester, dodecane or hexadecane as sole carbon source. For this purpose the cells were pre-cultivated in YPD for 24 h and then transferred onto the plates (5 μL, 5×10^6 cells/mL) and incubated for 30 h at 28°C. The blue colour of some colonies arising after this time without any other treatment indicated high expression of β-galactosidase activity. Following this first analysis the plates were exposed to toluene vapour for one minute and incubated at 37°C for additional 6 and 24 h to detect weak activities of β-galactosidase. The results in Table 2 demonstrate that the promoters of the genes *XPR2c*, *ICL1* and *G3P* were weakly active on glucose. In contrast, all other promoters were completely repressed during growth on glucose, glycerol or ethanol. Glycerol strongly induced the *G3P* promoter, whereas the *ICL1* promoter was very active on ethanol, oleic acid, ricinoleic acid methyl ester, and moderately induced on dodecane and hexadecane. As expected, the *POT1* and *POX2* promoters were not induced by glucose, glycerol and ethanol but highly active on oleic acid, ricinoleic acid methyl ester and on both tested alkanes. The *POX2* promoter seems to be highly induced on both alkanes whereas the *POT1* promoter, similar to the *ICL1* promoter, is only moderately active. In contrast, the *POX1* and *POX5* promoters were only weakly induced by ricinoleic acid methyl ester, dodecane and hexadecane. However, it has to be mentioned, that strain PO1d has, in contrast to other strains of *Y. lipolytica*, a very long lag phase after transfer of cells to alkane containing medium. Therefore, the expression levels of genes encoding enzymes of the β-oxidation and the gluconeogenetic pathway in strain PO1d on alkanes may not be representative for other strains of *Y. lipolytica*.

Table 2. Estimation of promoter activities by blue staining of colonies after cultivation on YNB+ different carbon sources for 30 h. RM = ricinoleic acid methyl ester, (–) no blue colour, (+) and (++) blue colour occurred after treatment with toluene after 24 h or (+++) after 3-6 h at 37°C, (++++) blue colour occurred without toluene treatment

Promoter	Glucose	Glycerol	Ethanol	Oleic acid	RM	Dodecane	Hexadecane
XPR2	–	–	–	–	–	–	–
XPR2c	+	+	+	+	+	+	+
ICL1	–+	+	+++	+++	++++	++	++
G3P	+	+++	+	+	+	+	+
POT1	–	–	–	+++	++++	++	++
POX1	–	–	–	–	–+	–+	–+
POX2	–	–	–	+++	++++	+++	+++
POX5	–	–	–	–	–+	–+	–+

pXPR2c was slightly induced by all carbon sources, as expected for a constitutive promoter, whereas *pXPR2* was not active on these media, at all.

In the second step of this study the time course of expression of β-galactosidase activity under the control of the different promoters was measured during cultivation of transformants in liquid minimal medium. Dodecane and hexadecane were excluded from this investigation, because of the very long lag phase of PO1d transformants in media containing these carbon sources.

The transformants were pre-cultivated as described in Materials and Methods and used for inoculation of minimal medium with either glucose, glycerol, oleic acid or ricinoleic acid methyl ester. Cell density and β-galactosidase activity were measured each hour for a period of 12 h (Fig. 2(a), (b)). This study showed that also high activity of β-galactosidase does not affect growth (data not shown). However, growth on ricinoleic acid methyl ester was restricted, because of accumulation of γ-decalactone, which inhibits growth [33].

In contrast to the plate assay the *G3P* promoter was not highly induced during cultivation in glycerol containing medium (Fig. 2(a)). The *G3P* promoter constructions differed in their expression levels. *pG3PB2*, which contains the intron of *G3P*, is about two times stronger on glucose, glycerol and oleic acid than *pG3PB1*. These data suggest that the intron could have

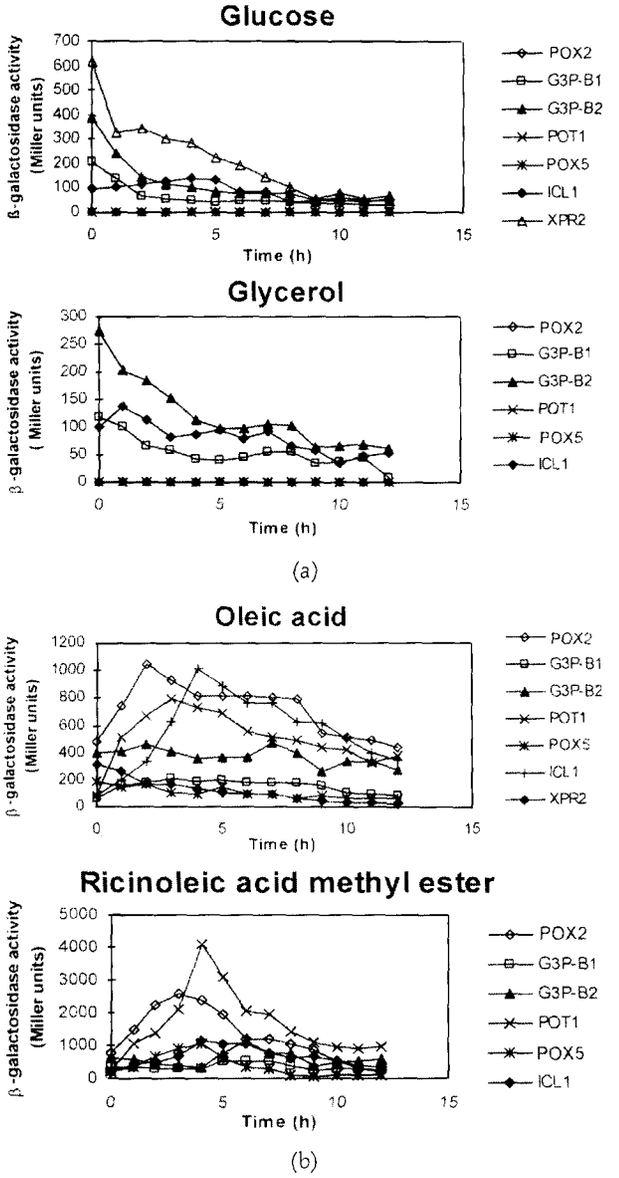

Fig. 2. (a) Expression of the reporter protein β-galactosidase in single copy transformants of *Y. lipolytica* PO1d under the control of different promoters in non-inducing conditions during cultivation on glucose and glycerol. The β-galactosidase activity was calculated as Miller units. The abbreviations mean that promoters of following genes were fused to the *lacZ* gene: *POX2, POX5* = acyl-CoA-oxidase genes, *G3P*-B1 = glycerol-3-phosphate dehydrogenase gene (*lacZ*-fusion at first ATG of the ORF), *G3P*-B2 = glycerol-3-phosphate dehydrogenase (*lacZ*-fusion after the intron of the *G3P* gene), *POT1* = 3-oxo-acyl-coA-thiolase gene, *ICL1* = isocitrate lyase gene (fusion with *lacZ* after the intron of *ICL1*), *XPR2* = alkaline extracellular protease gene (constitutively expressed hybrid promoter *XPR2c*), (b) Expression of the reporter protein β-galactosidase in single copy transformants of *Y. lipolytica* PO1d under the control of different promoters in inducing conditions during cultivation on oleic acid or ricinoleic acid methyl ester. Abbreviations see Fig. 2(a).

an enhancing effect on expression level. However, this effect was not studied in detail during this investigation, because of the moderate induction levels of both *G3P* promoter constructions at all tested carbon sources.

The *POX5* promoter was only weakly induced by oleic acid and the *XPR2* promoter was not active, at all, confirming the results of the plate assay.

Ricinoleic acid methyl ester very strongly activates the *POT1* and *POX2* promoters giving the highest activities after 3 to 4 h of cultivation (Fig. 2(b)). These results present ricinoleic acid methyl ester as the strongest inducer for *pPOX2* and *pPOT1* among the tested carbon sources, in spite of the disadvantage, that this carbon source inhibits growth of *Y. lipolytica*. Further studies will elucidate whether ricinoleic acid methyl ester can be used as strong inducer for heterologous gene expression under control of the *POT1* or the *POX2* promoters in mixed substrate fermentations or whether any effects will inhibit enzyme overproduction under such conditions.

Oleic acid is the carbon source without any growth inhibitory effect among the tested ones, which induced the highest activation of the promoters of the *ICL1* and *POX2* genes and to a little less extend of the *POT1* promoter (Fig. 2(b)). Similar results were obtained with the *ICL1* promoter on ethanol or acetate as sole carbon sources (data not shown). The highest values of -galactosidase activities of about 1000 to 1050 Miller units were measured under control of the *ICL1* and *POX2* promoters and of about 800 Miller units with the *POT1* promoter on oleic acid. These activities are higher than the activity with the *XPR2* promoter under inducing conditions, which do not exceed 540 Miller units [17].

Summarizing this study elucidated that the *POX2, ICL1,* and *POT1* promoters are the strongest available promoters for *Y. lipolytica*, at present. These promoters have the advantage that they are inducible by cheap carbon sources in minimal media, what improves also the purification procedures for secreted proteins. The *POX2* and *POT1* promoters are only inducible with fatty acids, fatty acid derivatives or alkanes, what may be, at least in some respect, a disadvantage for biotechnological applications. The *ICL1* promoter is additionally inducible by acetate or ethanol but is, in contrast to *pPOX2* and *pPOT1*, not completely repressible by glucose or glycerol. Further studies using these promoters inserted in the newly developed multiple copy integration vectors [12,13] have already been started to elucidate the potential of *Y. lipolytica* for heterologous gene expression. First results of successful high expression of the bovine cytochrome P45017α cDNA (*CYP17*) and homologous NADPH-cytochrome P450 reductase under the control of the *ICL1* promoter [28,34] as well as of the homologous lipase gene *LIP2* with the *POX2* promoter (Nicaud, unpublished) demonstrate that these promoters are very useful tools for induction of high expression levels in *Y. lipolytica*.

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