Construction of a recombinant wine yeast strain expressing a fungal pectate lyase gene

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Abstract

A gene fusion between the Saccharomyces cerevisiae actin gene promoter and the cDNA of the Fusarium solani f. sp. pisi pelA gene has been constructed. This expression cassette has been introduced into the industrial wine yeast strain T73. The resulting recombinant strain is able to secrete active PELA enzyme into the culture medium. In preliminary microvinification experiments the wine produced by this pectinolytic strain is indistinguishable from wine produced using the non-transformed strain on the basis of the chemical analyses. Large scale fermentations need to be carried out in order to assess the effects on filtrability.

Keywords: Fusarium solani f. sp. pisi; pelA; Pectate lyase; Wine yeast

1. Introduction

Enzymic breakdown of pectin occurs by the action of (i) pectin esterases, which release the methyl ester groups from pectin, and (ii) depolymerases, such as pectate or pectin lyases and polygalacturonases. Commercial pectinolytic enzymes, used in winemaking and obtained from fungal cultures, are used to improve juice extraction or wine clarification and filtration [1]. Fungal genes encoding pectate or pectin lyases have been cloned and sequenced from Aspergillus niger [2,3], Fusarium solani f. sp. pisi [4] and Glomerella cingulata [5]. The latter has also been expressed in yeast [5].

We selected a wine yeast strain, T73, from Alicante musts [6] and using mitochondrial DNA restriction profiles, demonstrated the predominance of this strain in industrial wine fermentations [7]. This work makes it practicable to modify winemaking by genetic engineering. Recently, we have constructed a recombinant T73 strain, which secretes a Trichoderma longibrachiatum β-(1,4)-endoglucanase, producing a wine with an increased fruity aroma [8]. In this paper we report the construction of a T73 strain expressing the previously cloned F. solani f. sp. pisi pelA gene encoding a pectate lyase (PELA). The secretion of the active enzyme by this recombinant strain and its ability to produce wine in the same way as the untransformed strain are also described.
Methods

Strains and culture conditions

The Saccharomyces cerevisiae industrial wine yeast strain T73 (CECT1894) has been commercialized by Lallemand Inc., (Montreal, Quebec, Canada). Its selection [6] and molecular characterization [9] have been described previously. For cultures, the yeast strain was grown on YPD (1% yeast extract, 1.5% peptone, 2% glucose, each w/v), SC (Difco Yeast Nitrogen Base without amino acids and with 2% w/v glucose) or SCA (SC supplemented with 1.5% w/v casamino acids). Cycloheximide was used at 0.5 μg/ml to maintain the transforming plasmids in yeast cultures. All the bacterial experiments were performed with Escherichia coli DH5α by standard procedures [10].

DNA manipulations

E. coli plasmid isolation and general DNA manipulations were carried out using standard protocols [10]. S. cerevisiae DNA isolation was done as described by Fujimura and Sakuma [11].

Transformations

E. coli transformations were done as described by Hanahan [12]. Yeast transformation was performed using the lithium acetate protocol of Gietz and co-workers [13] with the following modifications: after the heat shock, cells were resuspended in 1 ml of YPD, incubated at 30°C for 2 h with shaking, and then plated on selective YPD plates.

Construction of the pPL7 plasmid

The construction of plasmid pPL7 is shown in Fig. 1 and summarized as follows. The EcoRI-EcoRI fragment, which contains the F. solani f. sp. pisi pelA cDNA, was recovered from plasmid pPL1 [4] and cloned into the EcoRI site of pBlueScript II SK+ yielding plasmid pPL3. A S. cerevisiae DNA fragment containing the actin gene promoter [14] was synthesized by PCR using the M13 reverse primer, the oligonucleotide ACTSAC1 (5'-GGGACCCGGGTAGCTGCT-3') and PELA1 (5'-TGACGCCAGCACAGCTTG-3') as primers, 1 μl of a 1:50 dilution of the DNA solution obtained as described above, and 1 U of Dynazyme. Amplification consisted of 30 cycles of 30 s at 95°C, 45 s at 56°C, and 1 min at 72°C.

Enzymatic assays

Plate assays for pectate lyase production were done on YPD selective plates overlayed with 5 ml of 50 mM Tris.HCl, pH 8.5, 1 mM CaCl, 0.5% polygalacturonic acid sodium salt (Sigma; St. Louis MO, USA) and 0.7% agar. After solidification, plates were inoculated and incubated at 30°C for three days. Degradation halos were visualized by staining with 0.05% ruthenium red (Sigma) for 20 min, followed by washing with water. Pectate lyase activity in culture supernatants was assayed by measuring the increase in A235 as described in [16]. One unit of pectate lyase activity releases 1 μmol of unsaturated product per minute (ε235 = 4600 M⁻¹ cm⁻¹). Isocitrate dehydrogenase and hexokinase activities were measured as described in [17] and [18], respectively.

Cellular location

Transformants were grown in 30 ml of selective YPD medium and incubated at 30°C with orbital shaking for 24 h. Cells were collected by centrifugation, resuspended in 30 ml of selective SC medium to an A600 of 2.0 and re-incubated under the same conditions for 3 days. 5 ml samples were taken every 24 h. Cells were centrifuged, washed with distilled water, resuspended in 0.5 ml of 10 mM Tris.HCl pH 7.5, and kept on ice. 1 ml of 0.45 μm zirconia/silica
beads was added to the samples and the cells disrupted by shaking in the Mini-BeadBeater using three 20-s pulses at 5000 rpm with 1 min resting time on ice between pulses. Cell extracts were separated from debris by centrifugation at 13,000 \( \times g \) for 15 min at 4°C. Equivalent volumes of supernatant and cell extracts were assayed for pectate lyase, hexoquinase and isocitrate dehydrogenase activities.

**Microvinification experiments**

Fermentation assays were carried out using 1 litre of sterilized Muscat grape must (Teulada, Alicante, Spain) as previously described [6]. Vinification processes were stopped after 28 days, the wines centrifuged to remove yeast cells and subsequently transferred to new bottles, which were kept at 4°C until analysis. Analysis of the enological parameters were as described previously [6].

**Results and discussion**

**Construction of a pectinolytic wine yeast T$_{73}$ strain**

Plasmid pPL7 was constructed as described in Methods (see Fig. 1). In this plasmid the cDNA of the *F. solani* *f. sp. pisi* pelA gene is under the control of the *S. cerevisiae* actin gene promoter. The sequence upstream of the ATG start codon is of fungal origin but resembles the consensus sequence required for optimum expression in yeast [19]. The region is rich in As and poor in Gs, the latter being absent from the seven bases that precede the ATG codon, and an A appears at position 3. Cells of the industrial wine yeast strain T$_{73}$ were transformed with pPL7. After two days of growth several transformants arose on the selective plates containing cycloheximide.

Thirty of these transformants and one YEpcR21 transformant as a negative control were assayed for pectate lyase production using the overlay assay technique described in Methods. Only pPL7 transformants produced halos of degradation of polygalacturonic acid in the plate assay (results not shown). The presence of pPL7 in two of these transformants was demonstrated by PCR (see Fig. 2), indicating that the pectinolytic phenotype of these strains was due to the expression of the *F. solani* pelA gene.

**Secretion of the *F. solani* PELA pectate lyase in the industrial wine yeast T$_{73}$ strain**

Direct pectate lyase assays from YPD culture supernatants was unsuccessful because of colour interference by the medium at the wavelength assayed. For this reason, the untransformed T$_{73}$ strain, a YEpcR21 transformant, and eight pPL7 transformants were pre-grown in selective YPD medium for 24 h at 30°C with orbital shaking and then centrifuged, washed with sterile water and transferred to selective SC medium at a final A$_{600}$ of 2.0. Incubation was continued at 30°C with orbital shaking. Samples were collected every 24 h and assayed for cell density and pectate lyase activity. As can be seen in Table 1, all eight transformants secreted active pectate lyase into the culture medium.

Previous results, using an expression cassette comprising the *S. cerevisiae* actin gene promoter and the cDNA of the *T. longibrachiatum* egII gene, demonstrated the secretion of the fungal protein constitutively from early stages of the culture [8]. However, an apparent delay in enzyme secretion was detected in all the pelA transformants, since at 24 h they were entering the stationary phase (except PL-25), but only small amounts of pectate lyase activity were detected (Table 1). This delay may be related to cell lysis or secretion problems. To investigate this point further, the most actively secreting transformant (PL-1) and an average one (PL-21) were selected for analysis. PELA cellular location was examined in both transformants as described in Methods. As can be seen in Table 2, pectate lyase activity was mainly detected in the culture supernatants. The intracellular markers, isocitrate dehydrogenase and hexoquinase, were detected inside the cell only, except for 72 h cultures, in which neither hexoquinase nor isocitrate dehydrogenase were detected. Hence, PELA is actively secreted by the recombinant wine yeast and there is no cell lysis which could account for the detected activity. In order to establish whether the lack of secretion during the exponential growth phase is medium-dependent, we tested pectate lyase activity in cultures with the richer medium SCA. In this medium extracellular pectate lyase activity was detected during exponential growth, though the highest values were obtained in stationary phase (Table 3). As the *F. solani* PELA protein comprises only 242 amino acids, of which 12 are cysteines, some
Fig. 1. Strategy used for the construction of plasmid pPL7. Details are presented in Methods.
Problems related with protein folding may explain this apparent delay. An approach to solving this type of problem has been reported by Robinson and co-workers, who found overexpression of the yeast protein disulfide isomerase can improve the secretion of cysteine rich proteins by improving the folding [20].

Wine fermentations

Microvinification experiments were carried out with the untransformed T\textsubscript{73} strain and the YEpCR21, PL-1 and PL-21 transformants. After three days of fermentation, aliquots were taken to determine cell concentration and plasmid stability, T\textsubscript{73} and PL-1 fermentations had undergone eleven generations, while YEpCR21 and PL-21 fermentations only underwent eight generations. Plasmid stabilities, determined as the percentage of cycloheximide resistant cells, were 87.9, 81.2 and 83.1\% for YEpCR21, PL-1 and PL-21 respectively. At the end of the fermentation, the percentage of cycloheximide resistant cells had declined sharply to 26, 8.0 and 22.5\% for YEpCR21, PL-1 and PL-21, respectively.

Reducing sugar concentration was followed as a marker of the fermentation progress. No significant differences at the end of the fermentations were found among the wines produced by the pPL7 transformants and those produced by T\textsubscript{73} and the YEpCR21 transformant. As can be seen in Table 4,
Table 3
Culture density and pectate lyase activity in two different pPL7 transformants grown directly in SCA medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Culture density (A₂₅₀)</th>
<th>Pectate lyase activity (mU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 48 72</td>
<td>24 48 72</td>
</tr>
<tr>
<td>PL-1</td>
<td>3.3 6.2 9.8</td>
<td>22.6 41.3 252</td>
</tr>
<tr>
<td>PL-21</td>
<td>3.8 7.1 10.0</td>
<td>69.1 43.5 81.7</td>
</tr>
</tbody>
</table>

The physico-chemical parameters analyzed in the four wines were very similar and the slight differences observed could be due to the slower rate of fermentation in the YEpcR21 and PL-21 microvinifications noted above.

A pectinolytic wine yeast strain has been constructed. This recombinant wine yeast secretes an active form of the *Fusarium solani* f. sp. *pisi* PELA into the culture medium. Recently, Laing and Preto-rius described the expression of a bacterial pectate lyase gene in a wine yeast but no information about wine production was reported [21]. To our knowledge, the results presented here constitute the first evidence of the expression of a fungal pectinase gene in an industrial yeast strain. Moreover, in microvinification experiments this pectinolytic strain is able to produce a wine with the same physico-chemical characteristics as that produced by the untransformed strain. Industrial pilot plant vinifications need to be carried out to confirm these observations and to further elucidate the effect of the secreted enzyme on the whole vinification process and in particular on filtrability. However, legal permission is mandatory before we can continue with this scale-up.

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References


