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Amino acid supplementation, controlled oxygen limitation and sequential double induction improves heterologous xylanase production by *Pichia stipitis*

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Abstract

Heterologous endo-β-1,4-xylanase was produced by *Pichia stipitis* under control of the hypoxia-inducible *PsADH2*-promoter in a high-cell-density culture. After promoter induction by a shift to oxygen limitation, different aeration rates (oxygen transfer rates) were applied while maintaining oxygen-limitation. Initially, enzyme production was higher in oxygen-limited cultures with high rates of oxygen transfer, although the maximum xylanase activity was not significantly influenced. Amino acid supplementation increased the production of the heterologous endo-β-1,4-xylanase significantly in highly aerated oxygen-limited cultures, until glucose was depleted. A slight second induction of the promoter was observed in all cultures after the glucose had been consumed. The second induction was most obvious in amino acid-supplemented cultures with higher oxygen transfer rates during oxygen limitation. When such oxygen-limited cultures were shifted back to fully aerobic conditions, a significant re-induction of heterologous endo-β-1,4-xylanase production was observed. Re-induction was accompanied by ethanol consumption. A similar protein production pattern was observed when cultures were first grown on ethanol as sole carbon source and subsequently glucose and oxygen limitation were applied. Thus, we present the first expression system in yeast with a sequential double-inducible promoter.

Keywords: *Pichia stipitis*; Heterologous protein expression; Regulated promoter; Aeration; Endo-β-1,4-xylanase

1. Introduction

Pichia stipitis is a respiratory yeast, which implies that no ethanol is formed during aerobic cultivation, independent of the sugar concentration in the medium [1–3]. Therefore, this yeast is especially suitable for heterologous protein production, since it efficiently converts the carbon source to cell mass. High cell mass concentration is desirable for heterologous protein production because this enables a high volumetric product yield [4].

Several heterologous expression systems have been described for *P. stipitis* including the regulated *PsXYL1* and *PsADH2* promoters [5–7] and the constitutive *PsTKL* promoter [8,9]. Regulated systems are advantageous for heterologous protein production, because the growth and the protein production phase can be separated [4].

The *PsADH2*-promoter represents a novel induction system for heterologous protein production by *P. stipitis*

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[7]. The promoter is induced by oxygen limitation [10–12] so that induction is achieved without addition of supplementary (sometimes expensive) compounds. Once the desired cell density is reached protein production is induced by reducing the oxygen supply. Since in technical processes the cell density is rather high [4], oxygen limitation will rapidly occur because of the high respiratory activity of *P. stipitis* [2].

Another factor that may influence heterologous protein production by the hypoxia-inducible expression system is the degree of oxygen limitation. *P. stipitis* grows well under fully aerobic conditions but growth is severely retarded by oxygen limitation. The level of aeration and the oxygen transfer rate during oxygen-limited conditions strongly influence the rate of ethanol production [13] and therefore possibly also the expression level of the *PsADH2* gene. We have previously demonstrated that protein production was much higher in slightly aerated cultures than under anaerobic conditions [7].

Amino acid supplementation has been demonstrated to improve the production of heterologous proteins by yeasts in defined media ([14–17]; Görgens, J.F., et al., unpublished). Excess amino acids significantly improved heterologous protein production by auxotrophic, recombinant baker's yeast *Saccharomyces cerevisiae* (Görgens, J.F., et al., unpublished), indicating that amino acid supplementation may also be important for other auxotrophic recombinant yeasts. The proteolytic degradation of heterologous protein products decreased by supplementation of the medium with individual amino acids, such as arginine and lysine [18–20] (Görgens, J.F., et al., unpublished), or complex mixtures, such as casamino acids (e.g. [21]). Moreover, the synthesis of amino acids results in a net production of reduced redox equivalents. Therefore the addition of amino acids might especially improve protein production in an oxygen-limited system, where the capacity for utilisation of redox equivalents is limited [22].

In the present study amino acid supplementation and aeration were investigated in a *P. stipitis* high-cell-density culture producing the heterologous endo-β-1,4-xylanase under control of the hypoxia-inducible *PsADH2* expression system. For the first time we demonstrated a sequential double-inducible expression system in *P. stipitis*.

2. Materials and methods

2.1. Strains and medium

The recombinant *P. stipitis* strain PJH53 (*trp5-10*, *his3-1*) [*pVPA2CaXLN*] containing the *Cryptococcus albidus XLN* gene [5] under the control of the *PsADH2* promoter [7] was used in all experiments. Solid growth medium for the recombinant strain was prepared as described [7,11]. The basic growth medium for liquid cultivation, containing KH_2PO_4, $(NH_4)_2HPO_4$, $MgSO_4 \cdot 7H_2O$, Yeast Nitrogen Base without amino acids, glucose or ethanol and tryptophan (400 mg l^{-1}) was prepared as described previously [7,23]. When indicated, a mixture of amino acids was added to the cultures (Table 1). Amino acids were selected to ensure representation of all groups of amino acid in yeast biosynthesis [24]. The composition of the amino acid mixture was designed on the basis of a rational approach [25], whereby 40–60% of the amino acids required for biosynthesis were supplied exogenously.

2.2. Heterologous endo-β-1,4-xylanase production

Protein production was performed in controlled batch fermentation using Braun Biotech (Melsungen, Germany) Biostat® fermenters with a total volume of 2000 ml and a working volume of 1500 ml. Fermenters were sterilised according to the producer's manual, containing distilled water (90% of the final volume). After cooling to 30 °C, 150 ml of a filter-sterilised 10-fold concentrate of the liquid cultivation medium was added during inoculation (see below). The cultivation temperature was 30 °C and the pH of the cultures was controlled at pH 5.0 by the addition of 2-M NaOH or 2-M HCl. The fermentation broth was agitated in the range of 350–600 rpm and aerated with a 0.5 l min^{-1} airflow (standard conditions). The level of dissolved oxygen was monitored with a dissolved oxygen probe

Table 1
Amino acid mixtures added to cultures of *Pichia stipitis* PJH53 after induction of β-1,4-xylanase production

Amino acid[a]	Final concentrations (mg l^{-1})[b]	
	Lowly aerated culture	Highly aerated culture
Alanine	679	971
Arginine	296	443
Asparagine	590	842
Glutamine	744	1116
Glycine	338	507

[a] Amino acids were selected to ensure representation of all the amino acid groups in yeast biosynthesis [24].
[b] Amino acid concentrations were estimated based on biomass formation, resulting in lower concentrations for the lowly aerated condition.