PRODUCTION OF METHIONOL AND 3-(METHYLTHIO)-PROPYLACETATE WITH YEASTS

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Abstract

The Ehrlich pathway, prevalent in yeast metabolism, converts amino acids to the corresponding higher alcohols which may be further transesterified to their acetate esters. High product concentrations can be achieved if the amino acid is the sole nitrogen source and present at high concentrations. The main conversion products of L-methionine are 3-(methylthio)-1-propanol (methionol) and 3-(methylthio)-propylacetate (3-MTPA), which both smell broth-like and reminiscent of meat and potatoes. Up to now there is no report about an industrially applied process for the production of natural methionol and 3-MTPA. Overexpression of the ATF1 gene under the control of a TDH3 promoter together with an optimization of the glucose feeding regime lead to product concentrations of 2.2 g L^{-1} 3-MTPA plus 2.5 g L^{-1} methionol. These are the highest concentrations reported up to now for the synthesis of these valuable aroma compounds.

Introduction

The aroma compounds 3-(methylthio)-1-propanol (methionol) and 3-(methylthio)-propylacetate (3-MTPA) both have a powerful odour reminiscent of soup, meat, onions and potatoes and are derived from the sulphur amino acid methionine [1]. Naturally they occur in many fruits, beer and malt whisky. In cheese they are important compounds of characteristic aroma profiles [2] whereas in beer and wine the methionine derivatives are considered as off-flavours [3].

The deliberate production of methionine derived flavours is rarely reported, and if so, mostly in the context of soy sauce production, as those flavours contribute essentially to the condiment's aroma [4].

The Ehrlich pathway, which plays an important role in the biological formation of methionine-derived flavours is prevalent in yeasts and is especially active if the amino acid is the sole nitrogen source for the organism. Figure 1 shows the reaction principle. The amino acid is transaminated and decarboxylated to the corresponding aldehyde which is then reduced to the higher alcohol. If alcohol acetyl transferase activity is present in the organism, the alcohol can be partially transesterified to the acetate ester.

In previous investigations with L-phenylalanine as the sole nitrogen source for yeast strains, rose-like flavour compounds had been produced very successfully. Maximum product concentrations of 26.5 g L^{-1} 2-phenylethanol and 6.1 g L^{-1} 2-

phenylethyl-acetate could be achieved by intensive optimization of bioprocessing parameters [5] [6] [7] [8].

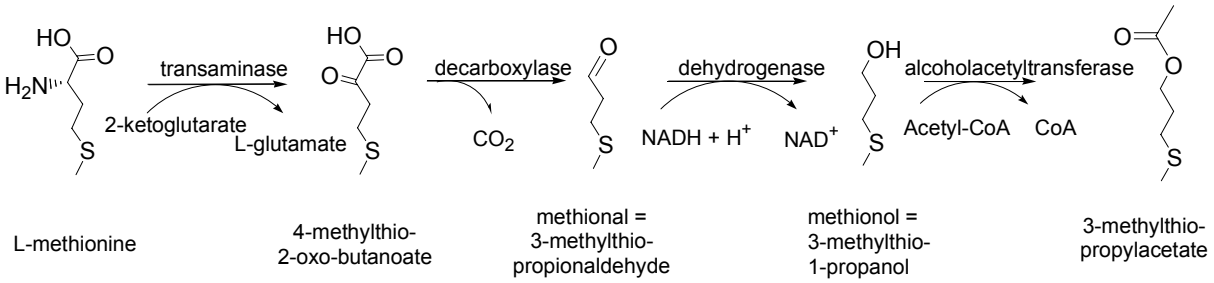

Figure 3 *Ehrlich pathway with L-methionine as precursor, leading from the amino acid to the higher alcohol with subsequent esterification to the acetate.*

In the present work metabolic engineering was applied in addition to the improvement of the bioprocess parameters for the cultivation of *S. cerevisiae* CEN.PK113-7D with L-methionine as nitrogen source. Overexpression of the *ATF1* gene coding for alcohol acetyl transferase 1 and adaptation of the glucose feed mode led to a considerably enhanced production of 3-MTPA. We therefore report for the first time the production of methionol and 3-(methylthio)-propylacetate on the grams per litre scale.

Experimental

For bioreactor and minireactor cultivations 20 g L^{-1} L-methionine, 12.4 g L^{-1} KH_2PO_4 and 1.6 g L^{-1} K_2HPO_4 were sterilized in deionised water. After cooling an appropriate amount of autoclaved glucose solution as well as 0.04 L of a filter-sterilized vitamin and trace mineral concentrate [6] was added per litre of medium. Cultivations were carried out at 30°C either in a 2.4 L bioreactor KLF 2000 (Bioengineering, Wald, Switzerland) or in a Dasgip FedBatch Pro 4-fold parallel cultivation system (Dasgip GmbH, Juelich, Germany). Process conditions and analytical procedures can be found elsewhere [9] in detail.

Results

Inhibition studies for *S. cerevisaie* CEN.PK113-7D with externally added methionol and 3-MTPA showed that growth impairment becomes noticeable only at concentrations of about 5 gL^{-1} and 2 gL^{-1} 3-MTPA respectively. In preliminary shake flask cultivations of *S. cerevisaie* CEN.PK113-7D the glucose reservoir was depleted after about 18 hours and 1.5 g L^{-1} methionol were synthesized during that time. As product inhibition effects were not to be expected at this concentration, measures for *in-situ* product removal were foregone for the time being. The acetate ester 3-MTPA was present, but at a concentration < 50 mg L^{-1}. By cultivation in a bioreactor carbon limitation was eliminated and process control improved. The process kinetics are given in Figure 2 and show that the formation of methionol started promptly and in a logarithmic fashion for the first 24 hours and shows association with the biomass formation. Both continued, albeit at a lower slope, until 64 hours. Apart from 6.2 g L^{-1} cell dry weight and 46 g L^{-1} ethanol, 3.5 g L^{-1} methionol were obtained in this fed-batch process. This corresponds to a yield of 0.64 mol mol^{-1} L-methionine and is

competitive with the only published bioprocess of industrial relevance whose product, however, is not methionol but its oxidation product 3-(methylthio)-propionic acid [10].

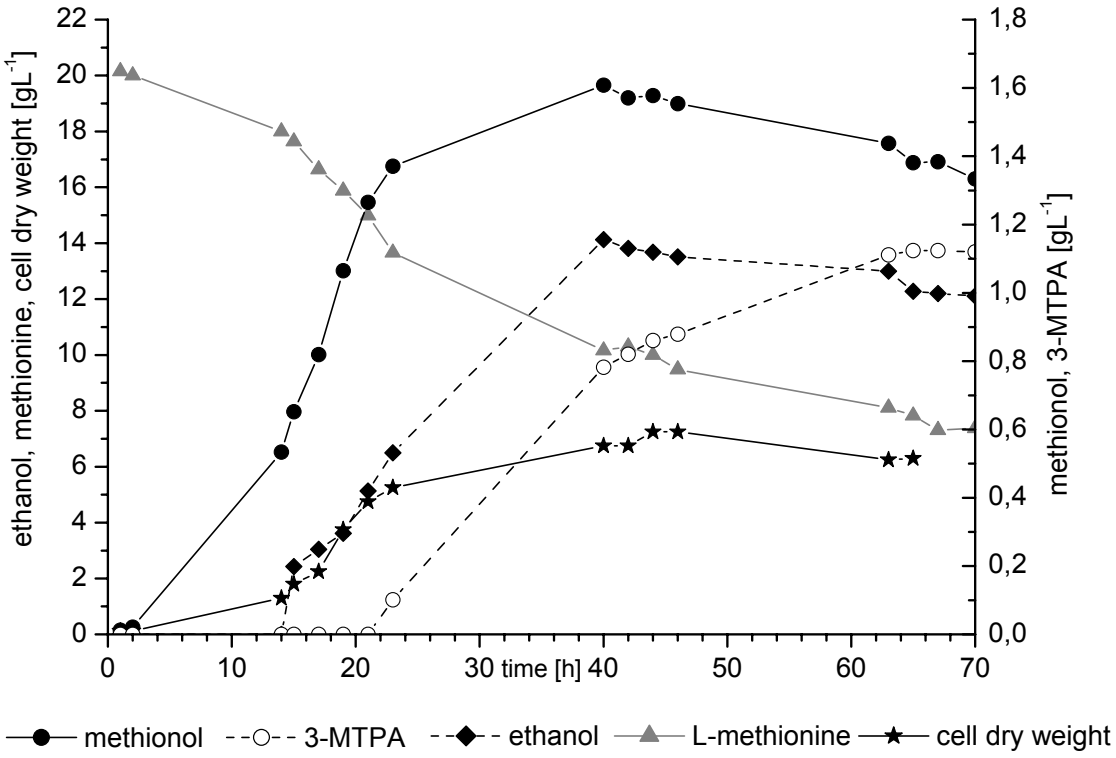

Figure 2. *Bioprocess kinetics of the production of methionol with S. cerevisiae CEN.PK113-7D during batch cultivation in a 0.45 L bioreactor with 0.2 L working volume. Glucose was fed at a constant flow rate between t= 26 h and 36 h. Reprint with kind permission of Springer [9].*

Strain optimization by genetic engineering. The existence of 3-MTPA in the cultivation broth showed that S. cerevisiae CEN.PK113-7D features at least one of the two alcohol acetyl transferase genes ATF1 and ATF2. The overexpression of the alcohol acetyl transferase gene ATF1 had been successfully proven before in wine yeast [11] and sake yeast [12]. However, due to the physiological amino acid concentrations in both grape and rice mash, the product concentrations in the examples cited above were naturally low. With the present work we investigated whether the principle of ATF1 overexpression could be transferred to S. cerevisiae CEN.PK113-7D and the Ehrlich pathway harnessed for efficient methionol-type flavour production in media with high amino acid concentration.

For this, strain CEN.PK834-1C was constructed by substitution of the *ATF1* promoter against the strong and constitutively expressed promoter of the *TDH3* gene. The kanMX4-TDH3p cassette was genomically integrated by double homologous recombination. The correct integration of both recombination sites was analysed by diagnostic PCR. Using primer pairs ATF1-A1/K2 and TDH3-A7/ATF1-A2 for the PCR resulted in the expected PCR products of 639bp and 319bp, respectively (data not shown).

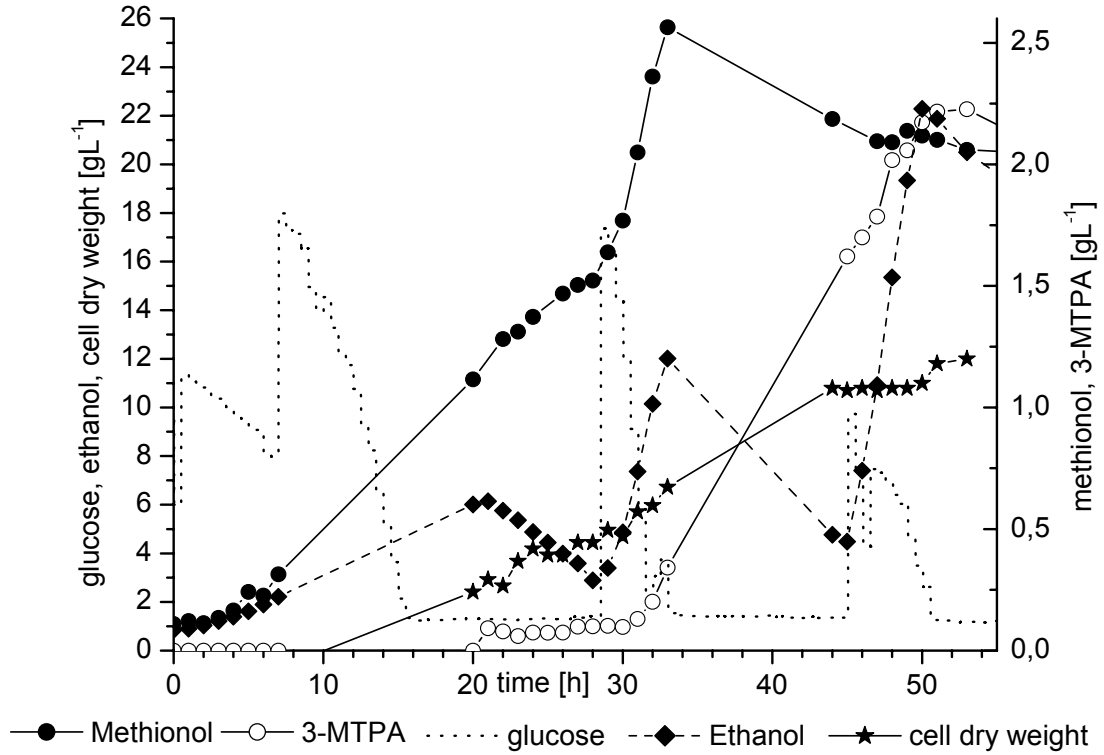

Figure 3. Bioprocess kinetics of S. cerevisiae CEN.PK834-1C in a bioreactor. Glucose was fed manually at t= 8 h, 29 h, and 45 h. The glucose concentration in the reactor was monitored online. Reprint with kind permission of Springer [9].

To avoid excessive ethanol accumulation depletion of the glucose reservoir was endorsed and thus the yeast forced to use ethanol as energy source. Under these conditions 2.2 g L^{-1} 3-MTPA were obtained in addition to 2.5 g L^{-1} methionol with the *ATF1* overexpression mutant (Figure 3). Concentrations of these methionol-type flavour compounds as high as these have – to the authors' knowledge - not been cited in literature before.

The 3-MTPA formation did not set in before a methionol concentration of > 1.0 to 1.5 g L^{-1} in the supernatant had been reached, indicating that not only overexpression but also a critical precursor concentration is necessary to thrive on Atf1 activity.

The shift from glucose as the sole carbon source to glucose and ethanol as alternate carbon sources caused a remarkable increase in product concentrations and yields $Y_{P/S}$ with CEN.PK834-1C compared to a bioreactor cultivation with a constant glucose concentration of 1-2 gL^{-1} (data not shown). The positive effect of the alternate C-sources is not explicable so far, thus further experiments are necessary to elucidate this phenomenon. Ideally these should include analyses of the cellular metabolite pools and of key enzyme activities involved in product formation to gain a better understanding of the bioprocess kinetics.