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Ethylene production by metabolic engineering of the yeast *Saccharomyces cerevisiae*

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ABSTRACT

The non-ethylene producing yeast, *Saccharomyces cerevisiae*, was transformed into an ethylene producer by introducing the ethylene forming enzyme from the plant pathogenic bacterium *Pseudomonas syringae*. Cultivation of the metabolically engineered strain was performed in well-controlled bioreactors as aerobic batch cultures with an on-line monitoring of ethylene production. The highest productivity was obtained during the respiro-fermentative growth on glucose but there was also a significant rate of formation during the subsequent phase of ethanol respiration. Furthermore, investigations were performed whether substitution of the original nitrogen source, NH_4^+, for glutamate could improve productivity and yield of ethylene even more. The rationale being that one of the substrates for the enzyme is 2-oxoglutarate and this compound can be formed from glutamate in a single reaction. Indeed, there was a substantial improvement in the rate of production and the final yield of ethylene was almost three times higher when NH_4^+ was replaced by glutamate.

1. Introduction

There is an increasing awareness that the supply of fossil fuels and especially oil is limited. As a consequence, prices are expected to show a sharp increase in the near future and this affects not only products like oil and gasoline but also all products derived thereof. Another complicating fact with the use of fossil material is the net CO_2 evolution with the potential effects on the global climate.

A very important group of products that rely almost entirely on fossil fuels as a feed stock are synthetic polymers including plastics such as polyethylene, polypropylene, polystyrene and polyvinyl chloride. In fact, ethylene is the organic compound that has the highest annual global production (McCoy et al., 2006). There is also a biological production as ethylene is an important plant hormone involved in signal transduction, for a review see e.g. De Paepe and Van der Straeten (2005) and Ecker (1995) but the amounts are of course quite limited. In addition to plants there are also a number of various micro-organisms that are able to synthesize ethylene (Fukuda et al., 1993). A great challenge for the community would be to replace chemical processes and the usage of finite fossil resources (e.g. for production of plastics) and instead develop sustainable biological systems using renewable substrates.

Biomass represents a carbon neutral renewable substrate that can be found in ample amounts. However, in order to be sustainable, cost effective and not to compete with the food market also lignocellulosic substrates such as trees, grass, plants and waste products from agricultural and paper industry as well as forestry waste has to be considered (van Maris et al., 2006). Unfortunately, lignocellulose is by nature quite resistant and it usually requires rather aggressive methods in order to liberate the fermentable sugars although more friendly methods using enzymatic hydrolysis are currently being developed. The aggressive methods used often provoke tough conditions also during the subsequent fermentation step with among other things a multitude of different growth inhibitors (Hahn-Hagerdal et al., 2006; Taherzadeh, 1999; van Maris et al., 2006). Baker's yeast, *Saccharomyces cerevisiae*, is offering many advantages as production organism in such environments. In general, it has a high tolerance to various inhibitors and there is an outstanding history of human usage and exploitation under large-scale industrial conditions. *S. cerevisiae* is, however, not a natural ethylene synthesizing organism. In order to transform the metabolism of *S. cerevisiae* into ethylene production we inserted the ethylene forming enzyme (EFE) from the plant pathogenic bacterium, *Pseudomonas syringae* (Fukuda et al., 1992; Goto et al., 1985; Nagahama et al., 1994) into a multicopyplasmid, pYX212, for expression in yeast. The EFE from *P. syringae* catalyzes the formation of ethylene as well as carbon dioxide and succinate from 2-oxoglutarate, arginine and oxygen (Fukuda et al., 1992).

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The aim of the project was to convert the non-ethylene producing industrial work horse, *S. cerevisiae*, into an ethylene producer by using a metabolic engineering approach. Furthermore, this work investigates the dynamics of ethylene production by on-line analysis during batch cultures performed under well-defined conditions in bioreactors. Finally, the influence of substituting the nitrogen source, ammonium, for glutamate, which can be converted to 2-oxoglutarate by a single reaction, was studied.

2. Material and methods

2.1. Yeast strains and plasmids

The yeast strain used was w303-1A (ura3-1). The open reading frame (ORF) of EFE was amplified by PCR, using the EFE-GUS construct (kindly supplied by M. Matsuoka); (Araki et al., 2000). An EcoRI site (underlined) was introduced immediately before the start codon (bold) in the upstream primer with the following sequence: GATCGAATTC**ATG**ACCAACCTACAGACTTTCGAG. The EcoRI site was later used to introduce the CIT1 mitochondrial targeting sequence immediately upstream of the start ATG. The primers used for cloning the CIT1 mitochondrial targeting sequence from *S. cerevisiae* were the following: CAGAATTCagatctATGTCAGCGATATTATCAACAACTAGCAA and CAGAATTCATAGTGGCGAGCATTCAATAGTGCAA. A BglII site (small letters) was also included downstream of the EcoRI site in the forward primer as control of correct orientation of the CIT1 peptide upstream the EFE sequence. The downstream primer (GATCAAGCT**TCA**ggatccTGAGCCTGTCGCGCGGGTGTC) introduces a BamHI site (small letters) immediately before the STOP codon (bold) and also introduces a downstream HindIII site (underlined) which overlaps one nucleotide with the stop codon. The PCR product was confirmed to be of a correct length, subsequently digested with EcoRI and HindIII and ligated into EcoRI/HindIII digested pYX212. The resulting plasmid was subsequently digested with BamHI and ligated with the BamHI fragment from either 313GHBwt or 313HBwt (Griffioen et al., 2000), resulting in a EFE tagged by GFP-HA or only HA-tag, respectively. The CIT1 N-terminal containing EFE was only tagged by GFP-HA. Correct inserts in all DNA-constructs were verified by sequencing. A complete sequence of the pYX212 (Ingenius, R&D systems, Madison, WI, USA) empty plasmid and pYX212-EFE are provided in the Web Supplement.

2.2. Medium and cultivation conditions

Cells were cultivated at 30 °C in a minimal medium, YNB w/o amino acids and ammonium sulphate (Difco) with 10 g/l of glucose as carbon and energy source and 5 g/l of either ammonium sulphate or glutamate as nitrogen source. Shake flask cultures were conducted in 100 ml bottles with a liquid volume of 10 ml placed on a rotary shaker operating at 120 r.p.m. During incubation the flasks were left open to allow aeration while at certain time intervals and different cell densities of a flask were made gas tight by sealing with a rubber stopper in order to record the amount of ethylene formed. Ethylene formation was stopped by putting the culture in dry ice and analysis was performed by using a GC (see below).

Bioreactor experiments were performed by using two identical SARA Fermentation systems (Belach Biotechnology AB, Bromma, Sweden). The liquid volume was 750 ml, pH was kept constant at 5.0 by automatic addition of 1 M NaOH, stirring rate was 600 r.p.m. and aerobic conditions were ensured by flushing with air at a rate of 150 ml/min. The bioreactors were connected on-line to a GC for analysis of evolved ethylene. Experiments using ammonium sulphate or glutamate as nitrogen source were each one performed in triplicates and the typical results presented in Figs. 2 and 3 were obtained by running the two experiments in parallel using the two identical fermentation systems simultaneously.

Results in Figs. 2 and 3 show representative results although all experiments were run in triplicates. Qualitatively, similar results were obtained but small variations in the time scale of the growth process prohibit a simple statistical representation including all individual results.

2.3. On-line analysis of ethylene by GC

The analysis of ethylene was performed by gas chromatography. The gas chromatograph was equipped with a gas sampling valve and a flame ionization detector. A packed porous polymer (HayeSepQ) column was used for the separation. The system was calibrated by analysing a standard gas mixture of 10 ppm ethene in nitrogen. The standard deviation of repeated analysis was on an average 3% of the absolute values.

Quantification of ethylene in the shake flask cultures was performed by penetrating the rubber stopper by a syringe needle connected to a gas switch. The switch was equipped with a 250 μl gas-loading loop that was filled with sample from the flasks and injected into the GC. This set-up enabled several replicate analyses from a single flask.

Ethylene formation in the bioreactors was analysed by connecting the off gas from the fermentors to the gas switch. The connected tubing and gas-loading loop were left open and flushed for 1 min (at a rate of 150 ml/min) in order to rinse the system before injection and GC analysis. This procedure was repeated three times at every sampling point.

2.4. Analysis of biomass concentration and extracellular metabolites

Dry weight was determined by centrifugation of 2×5 ml cell suspension at 2300*g* for 5 min. Pellets were washed two times and dried for 24 h at 110 °C. Samples for determination of extracellular metabolites were collected by centrifugation for 1 min at 16 000*g* and the supernatant was used for analysis using enzyme combination kits (Boehringer Mannheim, Mannheim, Germany).

3. Results and discussion

3.1. Construction of an ethylene producing strain of S. cerevisiae

The metabolic reactions carried out by the EFE enzyme is depicted in Fig. 1. The EFE expression plasmids, using the very strong TPI1 promotor from *S. cerevisiae*, were introduced into yeast strains (w303-1A *ura-* but all else prototrophic) by normal transformation and selection for uracil prototrohy. The transformed strains were subsequently tested for expression of the EFE-protein by western blotting and/or by fluorescence microscopy detection of GFP. Both the HA-tag and the GFP were in all cases easily detectable, thereby providing evidence for expression of protein from the plasmids. Furthermore, the western analysis indicated products of the expected size as predicted from the DNA-sequences. The strain expressing a mitochondrially targeted EFE, via the CIT1 N-terminus, showed a clear accumulation of intracellular fluorescent structures which correlated well with that observed using the dye mito-tracker (data not shown).

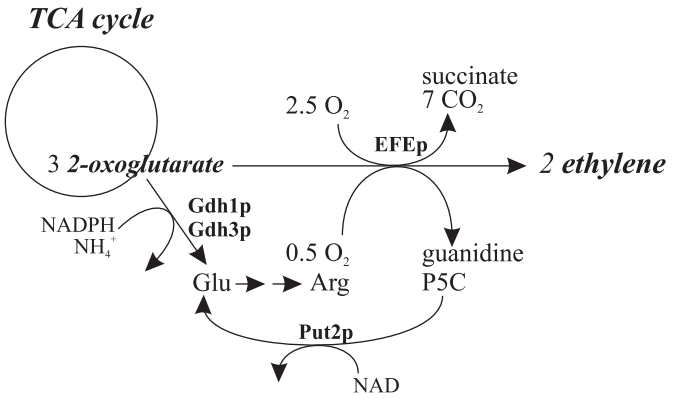

Fig. 1. Ethylene biosynthetic metabolism by insertion of the ethylene forming enzyme (EFE) from the plant pathogenic bacterium, *Pseudomonas syringae* (Fukuda et al., 1992) in the yeast *S. cerevisiae*.

The strains expressing the EFE constructs were subsequently tested for production of ethylene by culturing in gas-tight flasks followed by analysis of the retained gas. Comparing the wild-type yeast expressing an empty plasmid (control) to that expressing the non-tagged EFE-protein revealed a clear production only in the latter. The peak value, using glucose as carbon and energy source and ammonium as nitrogen source, was 240 μg/l h obtained at a fairly low cell density of approximately 1×10^7 cells/ml. It might be that the use of glucose causes repression/inhibition of important genes/proteins and thereby hampers production. Hence, to overcome this potential problem experiments were performed by substituting glucose for galactose, which should at least in part relieve the cells from repression/inhibition effects (Gancedo, 1998). However, repression/inhibition from glucose did not seem to be important since the ethylene production rates were drastically reduced when using galactose. Another attempt to improve the productivity that was more successful was to change the nitrogen source from ammonium to glutamate. The idea being that glutamate can be converted to one of the substrates for the EFE, 2-oxoglutarate, in a single step catalyzed by glutamate dehydrogenases (Fig. 1). Indeed, this almost doubled the maximum productivity of ethylene with peak values of 420 μg/l h at a cell density of approximately 1×10^7 cells/ml. Irrespective of nitrogen source, however, a declining ethylene production rate was observed during prolonged incubations, i.e. at cell densities above 1×10^7 cells/ml. Our interpretation of this phenomenon was that it is due to oxygen limitation. Not only will oxygen deficiency affects the overall energy metabolism of *S. cerevisiae* but it should also be kept in mind that oxygen is directly involved as a substrate in the ethylene forming reactions (Fig. 1). To avoid the problem of oxygen limitation and to quantify the dynamics of ethylene production during batch cultures shake flask experiments were abandoned in favour of high-performance bioreactors.

No ethylene production could be detected from the tagged version of the EFE protein or from the strains expressing a mitochondrial localization of the protein. The rationale for investigating the latter was that one of the substrates for the enzyme, 2-oxoglutarate, is produced in the TCA-cycle and hence a higher production of ethylene could be anticipated with its mitochondrial localization. It is difficult to know the reason for this disappointing result but one explanation could be that the supply of some of the other reactants involved might be limiting. Another reason for the lack of ethylene production in both the tagged and the mitochondrial protein could simply be that these were non-functional. Anyway, these strains were not subjected to further analysis.

3.2. Growth characteristics and dynamics of ethylene production in well-controlled aerobic bioreactors using ammonium or glutamate as nitrogen source

As expected the growth pattern of *S. cerevisiae* was not affected by the presence of the plasmid (cf. Henricsson et al., 2005; Larsson et al., 1998) and displayed the normal diauxic pattern irrespective of nitrogen source (Fig. 2). Initially, glucose is consumed by a respiro-fermentative metabolism with concomitant production of biomass as well as ethanol. Subsequently, once glucose is exhausted, respiration of ethanol results in yet another phase of biomass formation (Fig. 2). Although the same pattern is observed with both nitrogen sources the process is slightly delayed when using glutamate in comparison to ammonium. This is seen both as a lower rate of glucose consumption (Fig. 2) and as a lower specific growth rate, during glucose consumption (Fig. 3) similar to previous observations during anaerobic conditions (Albers et al., 1996). There was, however, an increasing biomass formation when using glutamate instead of ammonium (Fig. 2). The explanation being that glutamate does not only serve as a nitrogen source but the carbon skeleton can also be used as a potential carbon and energy source during aerobic conditions.

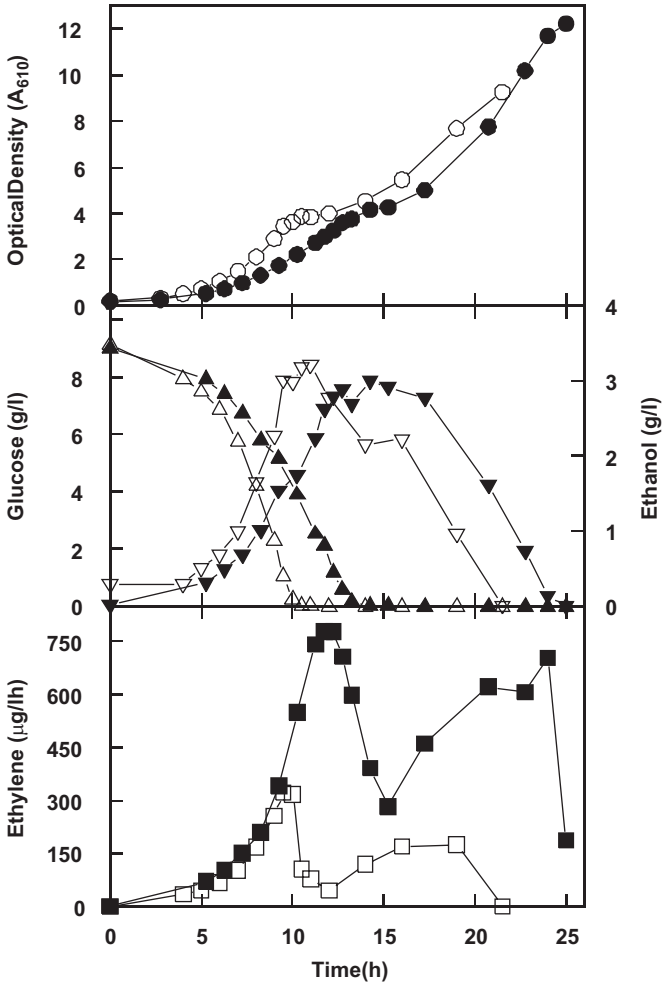

Fig. 2. Growth characteristics and ethylene formation in *S. cerevisiae* after insertion of the ethylene forming enzyme (EFE) from the plant pathogenic bacterium, *Pseudomonas syringae*. Cells were cultivated in minimal media with 10 g/l of glucose and either ammonium (open symbols) or glutamate (closed symbols) as the nitrogen source. Optical density (circles), glucose (triangles up), ethanol (triangles down) concentrations and ethylene production rates (squares) are shown. Cultures were run in triplicates and a representative result is shown.

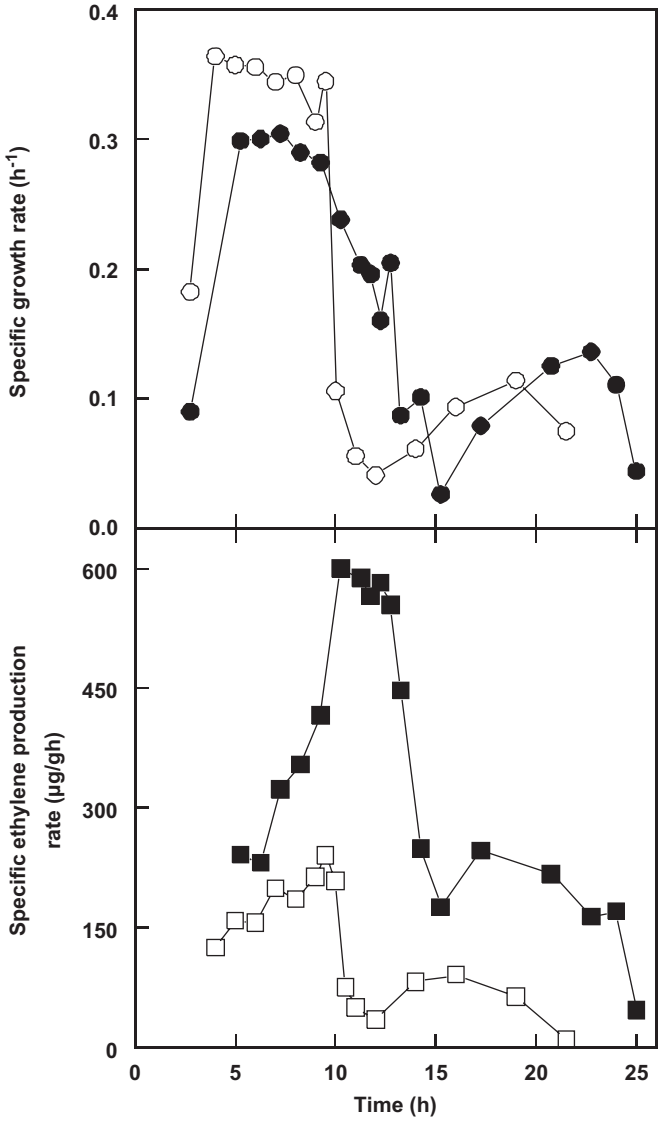

Fig. 3. Specific growth rate (circles) and specific ethylene production rate (squares) of *S. cerevisiae* after insertion of the ethylene forming enzyme (EFE) from the plant pathogenic bacterium, *Pseudomonas syringae*. Cells were cultivated in minimal media with 10 g/l of glucose and either ammonium (open symbols) or glutamate (closed symbols) as the nitrogen source. Cultures were run in triplicates and a representative result is shown.

Ethylene production showed a dynamic pattern throughout the entire cultivation period. There was an ever increasing production rate during the entire glucose consumption phase irrespective of nitrogen source with higher values compared to what was obtained in simple shake flask cultures (Fig. 2). Furthermore, the productivity was drastically improved when using glutamate instead of ammonium with a more than 100% increase in maximum production rate. Glucose depletion was accompanied by a sharp reduction in ethylene formation but this was followed by a continuous increase in productivity also during respiration of ethanol (Fig. 2). The highest levels were also during this phase obtained in the presence of glutamate and the proportional increase was even larger than observed during glucose utilization. It could be speculated that the increase in ethylene formation rate is simply a reflection of an increase in biomass concentration. This was, however, not the case since also the specific ethylene production rate showed increasing values during the entire glucose consumption period (Fig. 3). It was also found that the specific rate of ethylene formation was much higher during respiro-fermentative growth on glucose compared to respiratory growth on ethanol.

The superior productivity obtained when switching from ammonium to glutamate as the nitrogen source also provoked a much higher total yield of ethylene. Glutamate usage resulted in a total yield of 890 ± 160 μg ethylene/g glucose (SD, $n = 3$) while the corresponding value when using ammonium was only 320 ± 90 μg ethylene/g glucose (SD, $n = 3$). Statistical analysis by a two-tailed student's t-test revealed a significant difference at $p < 0.1$ using a mean standard deviation estimated from all data. In any case, however, there was only a small fraction of the available carbon that was diverted into ethylene production. The fraction being less than 0.1% using ammonium and about 0.2% with glutamate unless the carbon from glutamate is considered, this will cause also this value to drop below 0.1%.

To summarize, we have successfully engineered bakers yeast into an ethylene producer and by careful control of cultivation conditions managed to increase productivity as well as yield significantly. The highest productivity of 240 μg ethylene per litre and hour, obtained in shake flask cultures, was increased several fold by the use of well-controlled bioreactors and by substituting ammonium as nitrogen source in favour of glutamate.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ymben.2008.06.006.

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