Cloning, multicopy expression and fed-batch production of *Rhodotorula araucariae* Epoxide Hydrolase in *Yarrowia lipolytica*

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Abstract

Epoxide hydrolases (EHs) of fungal origin have the ability to catalyse the enantioselective hydrolysis of epoxides to their corresponding diols. However wild type fungal EHs are limited in the substrate range and enantioselectivity, additionally wild type fungal EH productivities are relatively low. Recombinant DNA technology has been previously used to overproduce these enzymes in expression systems such as *E. coli* and *A.niger and P. pastoris*. EH encoding genes from *Rhodotorula araucariae* were cloned and functionally expressed in *Y. lipolytica*, under the control of a growth inducible hp4d promoter. The transformation experiments yielded only two positive multicopy transformants, which were assessed in flask cultures. The selected transformant demonstrated a 4 fold enhanced EH activity over the transformant. The transformant was then evaluated in batch and fed batch fermentations, where the batch fermentations resulted in ~ 50% improved EH activity from flask evaluations. In fed batch fermentations, different specific feed rates were tested. A specific feed rate of 0.1 $g.g^{-1}.h^{-1}$ resulted in the highest EH activity of 1750 mU.mg dw^{-1}, compared to maximum production levels of 0.3 mU.mg dw^{-1} for the wild type R. araucariae and 52 mU.mg dw^{-1} *E. coli*. A 2.7-fold increase was observed from shake-flask fermentation to the fed-batch fermentation.

Introduction

Epoxide hydrolases (EH's) are enzymes that catalyse the *trans* addition of water to epoxides, resulting in the formation of the corresponding vicinal diols. EH's have been found in a wide variety of organisms, such as plants, bacteria, fungi, mammals, insects and yeasts and are hence regarded to be ubiquitous in nature (Weiijers et. al, 1999; Visser et. al., 2002 and Labuschagne et. al., 2006). Of these, soluble EH (sEH) and microsomal EH (mEH) are the best studied due to their broad substrate specificity and their role in the detoxification of epoxides derived from xenobiotic or endogenous compounds (Oesch, 1973). Stereochemical investigations have concluded that this attractive biocatalyst is responsible for the production of valuable chiral building blocks (enantiopure epoxides and corresponding enantiopure vicinal diols), which are used in the preparation of complex enantiopure bioactive compounds or as end products with biological activity (Krieg et. al, 2001, Weijers et. al, 1999; Visser et al., 2000; Botes et al. 1998).

Highly enantioselctive EHs have been found in bacteria (Archer et al. 1996; Ospiran et al. 1997) and fungi (Zhang et al. 1996; Grogan et al. 1996; Botes et al. 1998). A screening of 25 different yeast genera for the hydrolysis of 1,2 epoxyoctane resulted in the selection of *Trichosporon*, *Rhodotorula* and *Rhodosporidium* , which belonging to the bacidiomycetes genera and that displayed the asymmetric hydrolysis of the epoxide (Boets et al 1998) . The *Rhodotorula araucariae CBS 6031* isolate demonstrated an enantiomeric excess (e.e) values > 98% and enantiomeric ratio (E^e) >200 (Botes et al. 1998; Yeates et al. 2003). As a result, EH encoding genes from *Rhodotorula* have been expressed in *E. coli* (Visser et al. 2002) and the dimorphic fungus, *Yarrowia lipolytica* (Labuschagne et al. 2004; Labuschagne et al. 2006). The genes used for this study are as used by Visser et al., (2002)

In addition to these studies, other novel EHs were cloned from different fungi, including *Xanthophyllomyces dendrorhous* (Visser et al. 1999); *Aspergillus niger* (Arand et al. 1999) and *Rhodotorula glutins* (Visser et al. 2002).

Heterologous protein production using yeast as host is well documented (Russo M et al., 2005; Ramchuran et al., 2005; Kang et al., 1998. Recently *Y. lipolytica* has been developed as a suitable expression host for a variety of recombinant proteins (Nicaud et al., 2002). The potential capacity of this yeast for heterologous enzyme production has been demonstrated in a cyclic fed batch process strategy for the production of rice α-amylase (Chang et al., 1997). Furthermore, heterologous lipase activity of 90500 $U.mL^{-1}$ was achieved in fed batch fermentations using *Y. lipolytica* as an expression host and by increasing the number of copies of the *LIP2* gene under the control of the *POX2* promoter (Nicaud et al.2002). Therefore, it is not surprising that *Y. lipolytica* is emerging as an efficient host for the production of heterologous proteins. A wide range of selective markers, promoters, secretion signals and stable site directed or random integration of either single or multi-copy expression cassettes into genome are available for this expression system.

Labuschagne et al., 2004 and 2007, expressed EH encoding genes from *R. mucilaginosa* and *R. paludigenum* as single copy insertions into *Y. lipolytica* and demonstrated production levels only at flask scale. In this study we assessed both the multi-copy expression potential of *Y. lipolytica* using EH encoding genes from *R. araucariae* and its hetelogous protein production capability in flask, batch and fed batch fermentations. Furthemore, fed batch fermentation employing different specific nutrient feed regimes and evaluation the effect on enzyme production yields was conducted. To our knowledge this

work is first demonstration of multi-copy expression of EH encoding genes in *Y. lipolytica* and the evaluation of specific glucose feed rates on the production EH

Materials and methods

Strains and plasmids

The expression host *Y. lipolytica* Po1h was from Madzak et al. (2004) and expression vector pINA1291 was from Nicaud et al. (2002), the *eph* gene (epoxide hydrolase gene) was from *R. araucariae* (NCYC 3183). *Escherichia coli* TB1 (New England Biolabs, Beverley, MA, USA) was used as the host during plasmid construction. Restriction enzymes for DNA cleavage and ligation were from Fermentas (Vilnius, Lithuania) and used according to manufactures instructions. Oligonucleotides were synthesised by Integrated DNA Technologies (Coralville, IA, USA).

Vector construction

Plasmid pGEM-T:25 (Labuschagne, 2003) was digested with *Bam*HI and *Bln*I and the DNA fragments were separated by electrophoresis and purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Amersham, UK). The expression vector (pINA1291) was similarly digested with *Bam*HI and *Bln*I and dephosphorylated using Antarctic Phosphatase (New England Biolab's, Beverley, MA, USA). The *eph* gene and pINA1291 were subsequently ligated overnight at 4°C. The resulting plasmid was designated pYL25HmA (Figure 1) with the *R. aucariae eph* gene under the control of the growth-phase inducible hybrid hp4d promoter and transformed into *E. coli* TB1 using the Bio-Rad Gene-Pulser (Hercules, CA USA). Screening of the *E.coli* transformants was conducted by PCR screening using the following gene-specific primers:

25-fwd (19 mer): 5'-GTG GAT CCA TGA GCG AGC A-3'

25-rev (20 mer): 5'-GAC CTA GGT CAC GAC GAC AG-3'

Transformation of *Y. lipolytica* Po1h

Positive *E. coli* transformants were selected and cultivated overnight at 37°C in 50ml of LM (1% yeast extract, 1% tryptone, 0.5% NaCl) medium supplemented with 50 µg/ml kanamycin on an Innova rotary shaker (New Brunswick, Edison, NJ, USA) at 200rpm. Large-scale plasmid purification of pYL25HmA was carried out using Qiagen's Plasmid MIDI kit. The isolated plasmid DNA (5 µg) was digested with *Not*I to release the ~4 kb expression cassette, which was separated by agarose gel electrophoresis and purified similar to above. The cassette is bounded by the *zeta* regions and contains the *ura3d4* marker which is required in multiple copies to complement the auxotrophy of the host (Juretzek et al 2001) and the $hp4d_P$ - *eph* gene - $LIP2_T$ fragment to be integrated into the host genome. Transformation of *Y. lipolytica* Po1h was conducted according to Xuan et al. (1988). Colonies appearing on YNBcasa selective plates (2% glucose, 0.4% NH_4Cl, 0.2% casamino acids, 0.17% yeast nitrogen base without amino acids and without ammonium sulphate, 0.03% leucine, 1.5% agar) after 7 – 14 days were transferred onto fresh plates and sub-cultured.

Screening and clone selection

The *Y. lipolytica* transformants were subjected to genomic DNA isolation (Wizard® Genomic DNA Purification Kit, Promega, Madison, WI, USA) from overnight cultures grown in YPD (2% peptone, 2% glucose, 1% yeast extract). The genomic DNA (50ng) was subsequently used as the template for PCR screening using an Eppendorf Mastercycler

Gradient PCR machine (Hamburg, Germany) using 250 nM of each primer listed above at an annealing temperature of 50ºC for 1 min. The presence of PCR products of the expected size was taken as confirmation that integration of the relevant expression cassette had occurred in the genome of *Y. lipolytica* Po1h.

Positive clones were grown on selective agar plates (1.7% $m.v^{-1}$ yeast nitrogen base; 0.4% $m.v^{-1}$ NH_4Cl; 0.03% $m.v^{-1}$ leucine; 2.0% $m.v^{-1}$ glucose and 1.5% $m.v^{-1}$ agar) for 5 days. Clones were evaluated for recombinant EH activity by transferring a single colony into (100mL) liquid media (1.5% $m.v^{-1}$ yeast extract; 0.89% $m.v^{-1}$ malt extract and 0.67% $m.v^{-1}$ dextrose monohydrate) and cultivating flasks at 28^{o}C at 150 RPM (Innova 2300, New Brunswick Scientific, Edison, NJ) for 5 days. A final sample was taken and epoxide hydrolase activity using whole cells were measured.

Culture Maintenance and Inoculum

The selected clone was streaked on selective agar plates (1.7% $m.v^{-1}$ yeast nitrogen base; 0.4% $m.v^{-1}$ NH_4Cl; 0.03% $m.v^{-1}$ leucine; 2.0% $m.v^{-1}$ glucose and 1.5% $m.v^{-1}$ agar) and grown for 5 days, a single colony from this plate was inoculated into growth media (1.5% $m.v^{-1}$ yeast extract; 0.89% $m.v^{-1}$ malt extract and 0.67% $m.v^{-1}$ dextrose monohydrate) pre-sterilized for 20 min at 121^{o}C, and cultured for 24 hours at 28^{o}C at 150 RPM (Innova 2300, New Brunswick Scientific, Edison, NJ). Cultures were cryo-preserved using 25% $v.v^{-1}$ sterile glycerol as described by Meza et al. 2004. These cryo-preserved cultures were used as starter inoculate for all experiments by inoculating 1 cryovial into 700mL of growth media and cultured for 21 hours at 28^{o}C and 150RPM (Innova 2300, New Brunswick Scientific, Edison, NJ), before transferring to fermenters.

Evaluation of selected clone in Batch fermentation

All fermentation experiments were conducted in Braun C 15L fermenters (Braun, Germany). Duplicate fermentations, containing 9.3L of initial charge fermentation medium (as stipulated in patent WO/2007/010403), was inoculated with 700mL inoculum at an optical density (OD_{660nm}) of ~ 20 and a cell concentration of 1.82 X 10^8 ± 3.39 X10^7 CFU.mL^{-1}. The fermentation temperature was maintained at 28^oC ± 0.1, aeration at 1 vvm ± 0.003, impeller speed at 500 - 1000 rpm and pH at 5.5 ± 0.04 with NH_4OH (25% m.v^{-1}) and H_2SO_4 (20% m.m^{-1}). Dissolved oxygen was maintained above 25% saturation by varying impeller speed. Analysis of the fermentation exhaust gases were conducted using a Uras 10E, Hartman and Braun gas analyzer (Braun, Germany). Oxygen utilization (OUR) and Carbon dioxide evolution (CER) rates were calculated online by MFCS software (Braun, Germany.

Fed Batch Fermentation

The fed batch fermentation evaluation was conducted on the recipe and control parameters as for the batch fermentations. Four fermentations were fed a 60% m.m^{-1} glucose solution at varying specific feed rates, using a gravimetric feed controller to accurately maintain the desired feed rates. Sugar feed commenced when the residual glucose concentration dropped below 5 g.L^{-1}. Specific feed rates were calculated from an average dry cell weight total obtained from batch fermentation data and each fermentation was fed at different specific feed rates equating to 0.068, 0.085, 0.1 and 0.12 g.g^{-1}.h^{-1} respectively. Specific feed rate is a representation of the amount of sugar fed per gram of dry cell weight per hour (Hellwig et

al. 2000). The theoretical OUR at a specific feed rate of 0.12 $g.g^{-1}.h^{-1}$ was calculated to be 200 $mMol.L^{-1}.h^{-1}$, which is the near maximum limit obtainable in conventional stirred tank reactors at production scale. This was used as the basis for selection of the highest specific feed rate and varied at 20, 30 and 40 % increments below the maximum.

Sampling and analysis

Fermentations were sampled four hourly and analyzed for optical density at 660 nm using a Genesys 20 spectrophotometer (Spectronic, NY,USA) , dry cell weight (Jolivalt et al., 2005), glucose concentration using a Dionex HPLC (CarboPac^{TM} PA1 column, Dionex, MA, USA) and epoxide hydrolase enzyme activity.

Determination of Epoxide hydrolase activity

1,2- Epoxyhexane was selected as the substrate due to availability and it being considered as one of the preferred substrates for the selected clone (Lotter et al., 2000). Samples were assayed for volumetric epoxide hydrolase activity (µmole. $minute^{-1}$. mL^{-1}) towards 1,2-epoxyhexane (200mM). 1,2-Epoxyhexane was added to a final concentration of 200mM to 500µl cell suspensions (2.5% wet $weight.volume^{-1}$) in potassium phosphate buffer (50mM, pH 7.5 containing 20% $v.v^{-1}$ glycerol). The reactions were incubated (25°C) on an eppendorff shaker with gentle shaking. The reactions were initiated with the addition of substrate and allowed to react for 10 minutes; thereafter the samples were stopped with the addition of 500µl of ethyl acetate for extraction. The samples were vortexed for 30 seconds and centrifuged in a bench top centrifuge (Heraeus Biofuge Pico, Germany) at 13000 rpm. The organic fractions were dried over anhydrous magnesium sulphate and submitted for quantitative non-chiral 1,2-Epoxyhexane analysis by gas chromatography (GC).

Non-chiral GC for the quantitative analysis of 1,2-epoxyhexane bioconversion was performed on a Hewlett Packard 5890 series II gas chromatograph (GC) equipped with Flame Ionisation Detector (FID) and Agilent 6890 series auto sampler injector, using hydrogen as a carrier gas at a constant column head pressure at 10psi. The analysis of 1, 2 - Epoxyhexane was achieved using a non-chiral capillary GC column MDN 5S (Supelco) 30m length x 0.25mm internal diameter x 0.25µm film thickness, and 10mM 1- Heptanol (Sigma-Aldrich) as the internal standard. The injection temperature was maintained constant at 250°C, detector temperature at 300°C, and injection volume at 1.0 µL and split ratio at 50:1. The oven temperature programme was as follows: the initial temperature of 50°C was maintained for 1 minute, increased at a rate of 20°C per minute to 220°C, and maintained at this temperature for 2.5 minutes. The retention times (Rt) in minutes were as follows: 1,2-epoxyhexane = 2.8, 1-heptanol = 4.3 and 1,2-hexanediol = 5.0.

Data analysis and calculations

All fermentation data was subjected to stringent analysis and modelling. The 2nd order polynomial fit to fed batch dry cell weight totals, plotted against time, was significant, with $r^2 > 0.9$, the resultant quadratic equation was used to model the dry cell weight up to an age of 68 h (*Modelled Dry cell weight Total (g)* = $A\chi^2 + B\chi + C$), were χ = age (h). The dry cell weight and enzyme productivities were calculated as stipulated by Chang et.al. 1998 and Nori et al. 1983. The specific feed rates were calculated as described by Hellwig et al. 2000. Yield co-efficient calculations for dry cell weight and EH on sugar and oxygen was conducted as stipulated by Papanikolaou and Aggelis, 2001. Yield of product on sugar

(Y_{ps}), yield of product on oxygen (Y_{po}), yield of biomass on sugar (Y_{xs}) and yield of biomass on oxygen (Y_{xo}) were measured.

Results

Multicopy Transformation Y. lipolytica and evaluation of clones in shake flasks

Due to the low transformation efficiency of the *Y. lipolytivca* system (Madzak et al. 2004), only two clones designated YL1 and YL2 were obtained. The screening of these clones, based on recombinant EH activity, revealed a significant difference in enzyme activity levels at the end of the five day flask fermentation between the clones. The YL1-clone displayed the highest final EH activity of 6.82 ± 1.30 $mmol.min^{-1}.L^{-1}$, which was approximately 4-fold higher than the YL2-clone. Therefore, the YL1 clone was selected for further investigations and subsequently designated construct YL25.

Batch Fermentation.

Clone YL25 HmA was then evaluated in batch fermentation with an initial glucose concentration of 20% $m.v^{-1}$. A lag phase of ~ 5h was apparent after inoculation and the culture grew at a specific growth rate of 0.2336 h^{-1}. The highest dry cell weight concentration achieved during cultivation was 15.8 ± 1.7 $g.L^{-1}$ with the total volumetric biomass productivity of 0.91 $g.L^{-1}h^{-1}$ after 14.6 hours of cultivation (Fig.2). Although the maximum biomass titre (15.8 $g.L^{-1}$) was attained approximately 17.3 hours after inoculation, the initial glucose was depleted ~2.7 hours earlier (14.6 hours). At this stage of the cultivation the growth rate of the organism was apparently attenuated in response to substrate depletion as observed by the change in dry cell weight productivity (Fig. 2). A final volumetric cell mass concentration of 14.07 $g.l^{-1}$ was produced during batch

fermentation. With respect to enzyme activity, a maximum epoxide hydrolase activity of 13.93 ± 0.09 mmol.min^{-1}.L^{-1} was recorded 17.3 hours after inoculation, relating to a total volumetric enzyme activity of 14.12U.L^{-1}. This was approximately double the EH concentration measured in 700ml shake flasks (data not shown), thus indicating increased biomass titres being proportional to enzyme production yields.

Fed Batch Fermentation

The poor epoxide hydrolase activities observed in the batch fermentation led to the investigation of a linear glucose feeding strategy on the production of epoxide hydrolase enzyme. The dry cell weight total obtained from the batch fermentation were plotted and subjected to 2nd order polynomial analysis, the resultant equation ($0.2269x^2 + 2.5825x - 4.3775$) was used to predict the dry cell weight totals over a period of 68 hours. An age of 68 hours was theoretically calculated to be the age at which the operating volume of the fermenter would be exceeded at the highest feed rate. The average dry cell weight obtained over the entire modelled period was 443.4 g, which equated to a feed rate of 50.3, 62.8, 73.9 and 88.7 g.h^{-1}(as 60% glucose) for the designed specific feed rates of 0.068, 0.085, 0.1 and 0.12 respectively.

The oxygen utilization trends (Fig. 3a) observed for the fed-batch fermentations demonstrated an increased metabolic activity at increased specific feed rates. Figures 3b and c representing Dry cell weight and EH totals respectively clearly demonstrate increased totals for increase specific feed rates. In the feeding regime investigated, an increase in specific EH activity, dry cell weight and enzyme levels was observed in response to increased specific feed rates (Fig 4a, b and c respectively). The measured specific feed rates

were calculated from the average of the measured dry cell weights over the length of the fermentation and the measured glucose feed rates (Table 1). The measured specific feed rates were ~ 1.3 fold higher than the designed specific feed rates. This difference could reflect either inaccuracies in the model predictions, used to determine feed rates, or physiological differences between cultivation in batch and fed-batch culture. The relationship between specific feed rate and EH totals (Fig 4c) was linear ($y = 36022x - 1561.3$; $R^2 > 0.8$). Similar trends are apparent for specific feed rate and biomass totals ($y = 21330x - 956.16$; $R^2 > 0.9$) and specific EH activity ($3290.9x - 126.63$; $R^2 > 0.9$). The average yield of biomass on sugar (Y_{xs}) for the four fed batch fermentations was 0.57 $g.g^{-1}.h^{-1} \pm 0.02$, thus indicating that no change in Y_{xs} with changing specific feed rates.

Discussion

Labuschagne et al., 2004 and 2007, cloned EH encoding genes from *R.* paludgenum (2004) and *R.* mucilaginosa (2007) into *Y. lipolytica.* The cloning experiments were done soley as single copy insertions into the *Y. lipolytica* genome, additionally the demonstration of the functional expression of the EH encoding genes in *Y. lipolytica* was done at flask scale only. Visser et al., 2002, expressed the same EH encoding genes from *R. araucariae* as used in this study, in *E. coli*, as a single copy insert. The purpose of this study was to evaluate the multi-copy expression of EH encoding genes from *R. araucariae* (Visser et al., 2002), in recombinant *Y. lipolytica*, in batch and fed batch fermentation culture as well as a comparison between the EH production obtained in wild type *R. araucariae* and recombinant *E. coli*. Due to the lack of published information on recombinant expression of EH encoding genes from *R. araucariae* in *Y. lipolytica*, in both single and multi-copy, a direct comparison in EH production levels could not be conducted.

The flask assessment of the resultant clones led to the conclusive selection of a single clone demonstrating excellent activity toward the substrate 1,2 epoxyhexane, for further investigation in laboratory scale fermenters.

Growth-associated epoxide hydrolase production in batch fermentation was improved compared to shake-flask cultivation, although the final enzyme activity was still low, partly due to the short production period. The end of the batch fermentation was directly related to depletion of sugar, which was immediately followed by decrease in the product and biomass incremental productivities (Figure 2). The glucose was depleted at ~ 14h, although the production of EH continued for another 5 hours did not decline at this stage. The depletion of glucose would have triggered the early onset of the idiophase (Silva et al., 1998 and Moresi, 1994). The YL25 clone selected for investigation is under the strict control of the hp4d promoter, Nicaud et al., (2002) suggested that the hp4d promoter is a growth phase-dependent, with production accelerating at the entry into stationary phase (idiophase). It was therefore hypothesised if the idiophase was sufficiently delayed by additional feeding in fed-batch cultivation, growth-associated EH production and the specific EH activity could be drastically increased. In the fed batch fermentations, the initial glucose concentration was maintained at the same level as for the batch, to induce an early idiophase. Figure 3a demonstrates the drastic change in the slope of the OUR trend at ~ 13h which coincided with the depletion of glucose. Glucose concentrations in all fed batch fermentations after the start of glucose feed was zero, this ensured that the growth of the fermentation was controlled by the sugar feed rate.

Previous research on the production of lipase in recombinant *Y. lipolytica* under the control of the hp4d promoter demonstrated substantial increases in activities from shake-flask to batch to fed batch fermentations of 2000, 11500 and 90500 $U.mL^{-1}$ respectively (Nicaud et al., 2000), reflecting an increase in activity of almost 2 orders of magnitude. The results obtained during the present study demonstrated similar trends of 6.82, 13.96 and 132.43 $U.L^{-1}$ for shake-flask, batch and fed batch fermentations equating to ~ 20 fold increase from flask to fed batch. However there is currently no available scientific representation of the effect of specific feed rates on heterologous protein expression in recombinant *Y. lipolytica*.

The effect of the specific feed rate on specific EH activity, biomass formation, and final EH concentration, represented in Figure 4, demonstrate a highly linear relationship between all three variables and the specific feed rate. Increasing the specific feed rate from 0.08 to 0.1 $g.g^{-}1.h^{-1}$ resulted in an increase of ~ 68% in specific EH activity and ~ 75% in total EH production. As the residual glucose concentration remained zero during all fed-batch fermentations, cultures remained carbon-limited, while the increase in biomass production was directly translated into an increase in growth-associated EH production. Results indicate that EH production may be increased further by increasing the specific feed rates above maximum of 0.1 $g.g.h^{-1}$ tested. However, the OUR (Figure 3a) at a specific feed rate of 0.1 $g.g.h^{-1}$ reaches ~ 200 $mMol.L^{-1}$, which represents an upper limit in the scale-up of the process in a stirred tank reactor, due to limitations such as cooling and oxygen transfer rates (Ozturk, 1996 and Riviere, 1977). Figure 3b clearly demonstrates that with increased glucose feeds there is a related increase in biomass, this increased biomass requires substantial amounts of cooling to counter the heat generated during metabolism,

and therefore if specific feed rates are increased to greater than 0.1 $g.g^{-1}.h^{-1}$, the cooling requirements will not be met within conventional stirred tank reactors.

The best EH activity observed during this study was 1750 mU.mg dw^{-1}, attained in a fed batch fermentation fed at a specific feed rate of ~ 0.1 $g.g^{-1}h^{-1}$. Visser et al., (2002) demonstrated an EH activity of 0.3 mU.mg dw^{-1} for thw wild type *R. araucariae* and 52 mU.mg dw^{-1} for the recombinant *E. coli*, containing the EH encoding genes from *R. araucariae.* For the flask evaluation of the multi-copy expression of this study, an EH activity of 590.6 mU.mg dw^{-1} was observed, which was greater than 1 order of magnitude higher that observed by Visser et al. (2002) for recombinant *E. coli* bearing the same genes. This comparison clearly demonstrates the benefits of using multi-copy gene expression in *Y. lipolytica* system.

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Table Legends

Table 1. Fed Batch fermentation results for varying specific feed rates.

Figure Legends

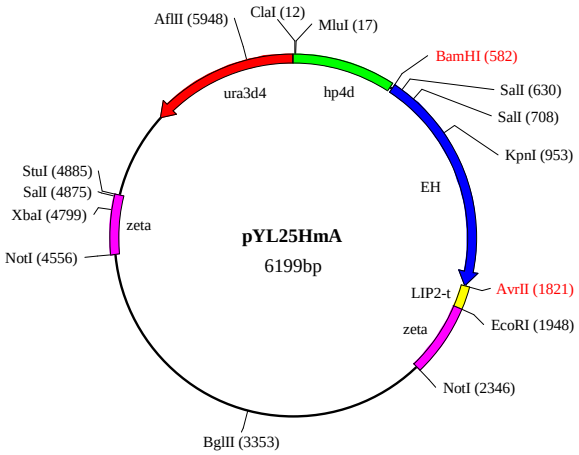
Figure 1. pYL25HmA, containing the *R. auracariae eph* gene (EH) under control of the hp4d promoter and LIP2 terminator (LIP2-t).

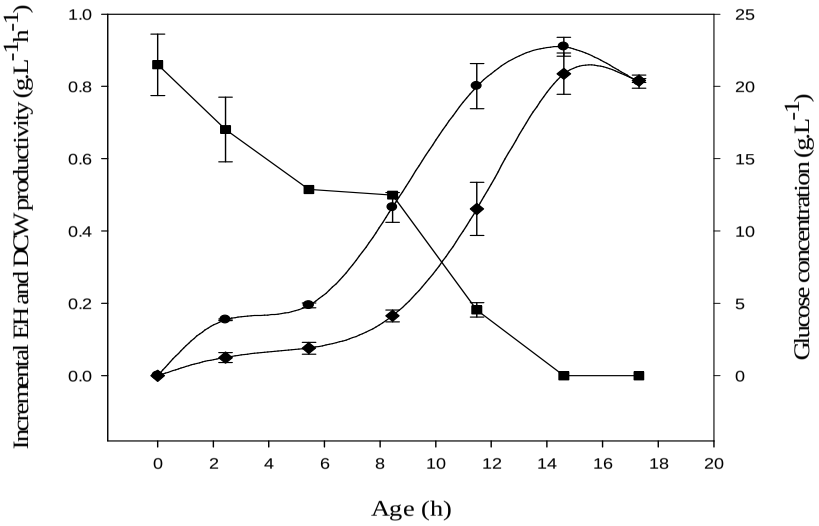
Figure 2. The incremental biomass, •, EH ,◆, productivities and glucose concentrations ,▪, of batch fermentation of *Yarrowia lipolytica*.

Figure 3. Fed batch fermentation profiles, **A** Oxygen utilization rates; **B** Total Biomass and **C** Total EH for the four test fermentations. F1 ———; F2 - - - - -; F3 ····· and F4 ——— · ·.

Figure 4. Effect of specific feed rate on, **A** Specific EH activity, **B** total biomass production and **C** total EH production.

Fermentation #	Measured Specific Feed Rates $g.g^{-1}.h^{-1}$	EH Productivity $U.L^{-1}.h^{-1}$	Biomass Productivity $g.L^{-1}.h^{-1}$	Y_{ps} $U.g^{-1}$	Y_{po} $U.g^{-1}$	Y_{xs} $g.g^{-1}$	Y_{xo} $g.g^{-1}$	Specific Activity $U.L^{-1}$	Total Enzyme U	Total Biomass g
F1	0.081	2.132	1.270	1.145	1.613	0.608	0.879	132.44	1437.08	782.64
F2	0.084	2.535	1.446	0.877	1.278	0.543	0.769	157.27	1324.26	820.27
F2	0.094	2.889	1.568	1.078	1.875	0.565	0.955	178.96	1889.04	990.15
F4	0.097	3.146	1.791	0.919	1.644	0.560	0.975	194.69	1910.12	1165.00


AflII (5948)
ClaI (12)
MluI (17)
BamHI (582)
SalI (630)
SalI (708)
KpnI (953)
ura3d4
hp4d
EH
StuI (4885)
SalI (4875)
XbaI (4799)
zeta
NotI (4556)
pYL25HmA
6199bp
LIP2-t
AvrII (1821)
EcoRI (1948)
zeta
NotI (2346)
BglII (3353)

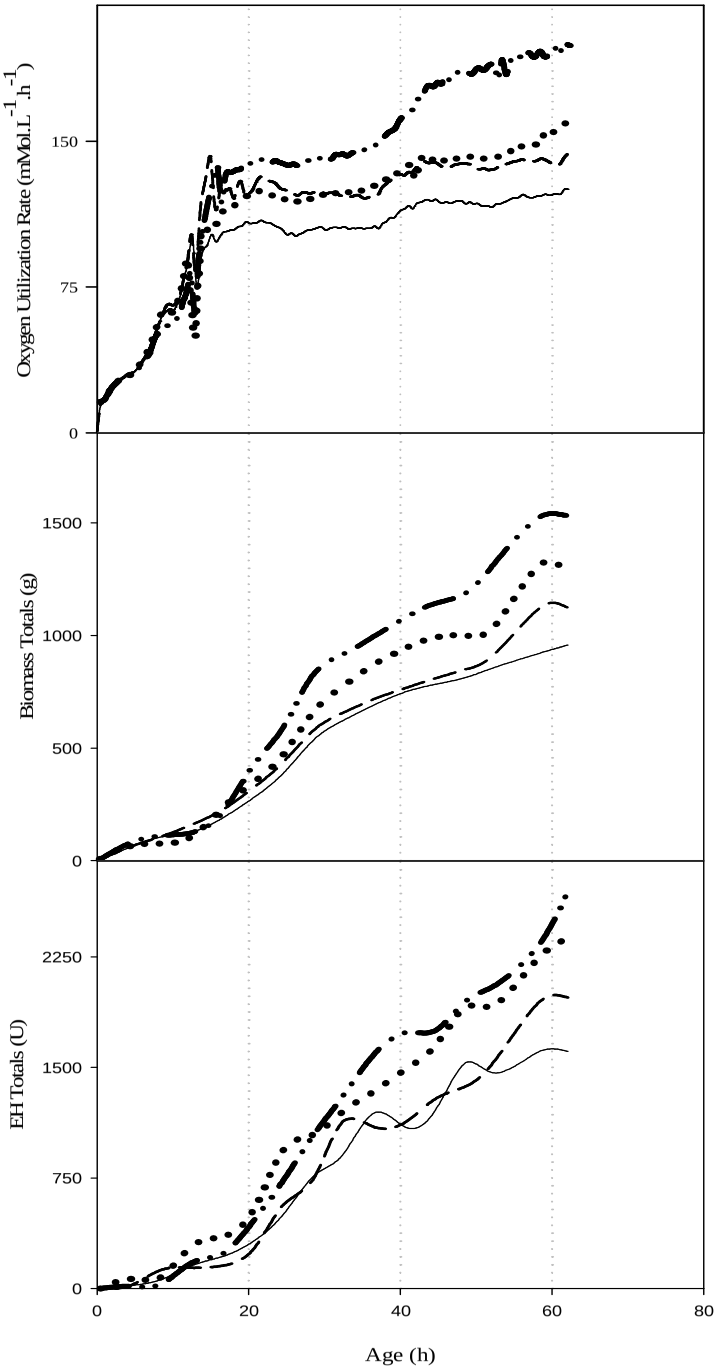

Incremental EH and DCW productivity (g.L-1h-1)
Glucose concentration (g.L-1)
Age (h)
1.0
0.8
0.6
0.4
0.2
0.0
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
18
20

1

2

3

4

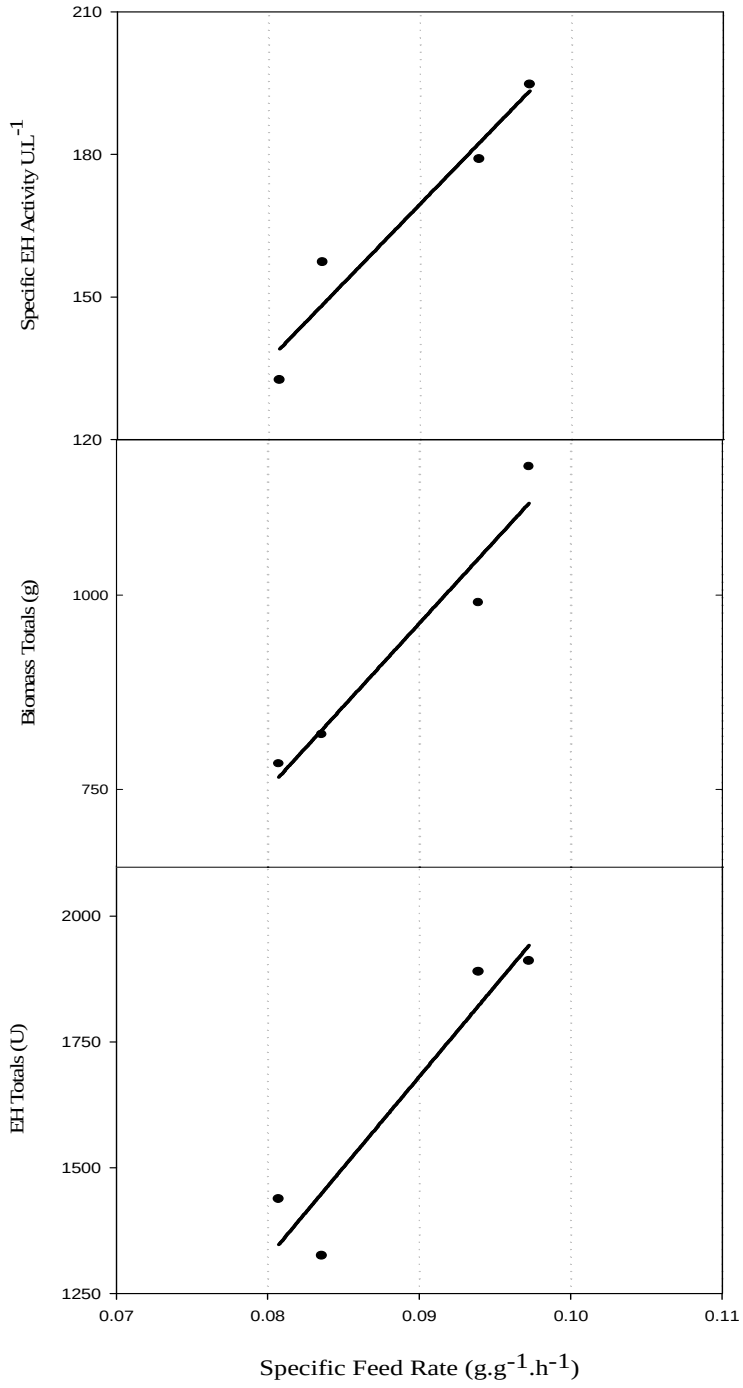

Oxygen Utilization Rate (mMol.L-1.h-1)
150
75
0
Biomass Totals (g)
1500
1000
500
0
EH Totals (U)
2250
1500
750
0
0
20
40
60
80
Age (h)

1

2

3

4


Specific EH Activity U.L-1
210
180
150
120
Biomass Totals (g)
1000
750
EH Totals (U)
2000
1750
1500
1250
0.07
0.08
0.09
0.10
0.11
Specific Feed Rate (g.g-1.h-1)

1

2

3