Recent advances in *Pichia pastoris* as host for heterologous expression system for lipases

Francisco Valero

(Francisco.Valero@uab.cat) Departament d'Enginyeria Química, Biològica i Ambiental. EE. Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.

ABSTRACT

The production of heterologous lipases is one of the most promising strategies to increase the productivity of the bioprocesses and to reduce costs, with the final objective that more industrial lipase applications could be implemented.

In this chapter, an overview of the new success in synthetic biology, with traditional molecular genetics techniques and bioprocess engineering in the last five years in the cell factory *Pichia pastoris,* the most promising host system for heterologous lipase production is presented.

The goals get on heterologous *Candida antarctica, Rhizopus oryzae* and *Candida rugosa* lipases, three of the most common lipases used in biocatalysis, are showed. Finally, new cell factories producing heterologous lipases are presented.

Keywords: microbial lipases, heterologous expression, bioprocess engineering, synthetic biology, *Candida rugosa, Rhizopus oryzae, Candida antarctica, Pichia pastoris*.

INTRODUCTION

Pichia pastoris is one of the most used cell factories for the production of many recombinant products. It can produce grams amounts per litter of recombinant protein both intra and extracellularly. However is not so easy predicting the amount of a target protein previous to experimental production (1). Trying to avoid an strategy of "trial and error" a comparative analysis of 79 different human cDNAs expressed in *P. pastoris* showed that low abundance of AT-rich regions in the cDNA were associated with a high expression level, high isoelectric point of the recombinant protein was associated with failure of expression and finally the occurrence of protein homologue in yeast was associated with detectable protein expression. Surprisingly, codon usage or GC content did not show a significant impact on protein yield (2). Thus, the prediction of a target protein expression level in *P. pastoris* is not easy.

From the previous review (3*) P. pastoris* cell factory has been improved to overcome the traditional problems associated to this methylotrophic yeast. The new approaches have been focused in two big areas, coupling the tools of synthetic biology with traditional molecular genetic techniques, and bioprocess engineering.

SYNTHETIC BIOLOGY

Current tools and future advances on synthetic biology and molecular genetics in *P. pastoris* and other non conventional yeast has been implemented (4).

The offer of new promoters as an alternative to the classical inducible AOX1 promoter and the constitutive GAP promoter has increased significantly in the last years. A summary of both, new inducible and constitutive promoters, can been consulted in the bibliography (1,5). A promoter collection can serve as an excellent toolbox for cell and metabolic engineering, and for gene expression to recombinant protein production. Studies of genome scale

transcriptomics are a valuable source of information of native promoters, and have been successfully applied to identify promoters of different strength and desired regulatory behaviour (6). The design of expression vectors has also been extended (1).

A comparative study with 48 different codon-optimized synthetic genes coding for the same protein under the control of four different promoters showed that a high gene dosage negatively influenced protein production in fed-batch cultivations (7).

The coexpression of enzymes which plays key role in the methanol utilization pathway also affected the specific growth and substrate rates and productivity (8).

The co-expression of folding and secretion factors (9-10), the optimization of the properties of foreign nucleotide sequence (codon usage) (11), the insertion of multiple recombinant gene copies (12), engineering of glycosylation (13) and secretory pathways (14) continue as topics to improve the performance of *Pichia* as cell factory (16).

Recently, the effect of hypoxia on the lipodome of *Pichia* has been also studied showed that cells adjust their membrane composition to oxygen limitation mainly by changing their sterol and sphingolipids composition resulting in higher recombinant protein secretion levels (16).

Since the development of surface-displayed functional proteins on microorganisms, cell surface display has been used in many fields (17). As a type of whole-cell biocatalyst, it not only can be used directly without purification and immobilization, but also can be reused for several times (18). Glycosylphosphatidylinositol (GPI)-anchored proteins are essential for viability and maintenance of normal cell morphology in yeasts (19). The knowledge of *Pichia* genome let to identify 13 GPI-modified cell wall proteins with the objective to express proteins anchorage on the cell surface (20). Therefore, the surface-displayed enzymes represent effective whole-cell biocatalysts which exhibit good potential in industrial bioconversion processes (21).

The development of specific systems biotechnology tools for *P. pastoris* has opened new opportunities for strain improvement and rational design of culture conditions (22-23).

The study of the properties of metabolic networks and their regulation *in vivo* is a key field in systems biology and, together with proteomic, transcriptomic or fluxomic studies has become a tool for strain optimization (24-25). Among the main *_omics* technologies, metabolomics and fluxomics are expected to lay a significant role in bridging the phenotype to genotype gap (26).

The determination of intracellular fluxes including cycles by means of ^{13}C-based metabolic flux analysis has been let the estimation of fluxes, including fluxes beyond central carbon metabolism (27-28).

BIOPROCESS ENGINEERING

From the point of view Bioprocess engineering important effort has been made in the field of process monitoring (5, 29). Fed-batch fermentation is the standard operational mode to produce recombinant proteins in *Pichia*. These strategies depend basically on the kind of the promoter selected. A summary of different fed-batch and continuous cultivations and process control strategies has been summarized (5). It is difficult discriminate if the physiological effects observed in a culture are due to the different cultivation strategies applied or the molecular effect of gene construct, making difficult the overall optimisation system (15). The relationship between specific growth, consumption and production rates, independently of the selected promoter, is nowadays a pivotal concept for bioprocess engineering of *P. pastoris* (15,30).

A fast and easy-to-do approach based on batch cultivations with methanol pulses has been successfully implemented to characterize different *P. pastoris* strains (8).

Important efforts have been made to reduce the time and the number of experiments to get the relationship between specific growth and production rates. In only a single fed-batch experiment the kinetics of product formation has been obtained in a novel synthetic variant of the AOX1 promoter (31).

Recent studies that purpose fully employed dynamic conditions, such as shifts, pulses, ramps and oscillations, for fast physiological strain characterisation and bioprocess development have been reviewed (32). Dynamic processes reveal information about the analysed system faster than traditional strategies, like continuous cultivations, as process parameters can directly be linked to platform and product parameters (32).

The specific substrate uptake rate (q_s) as a novel parameter to design fed-batch strategies for *P. pastoris* has been recently proposed. The dynamic feeding strategy where the feed was adjusted in steps to the maximum specific substrate uptake rate was superior to more traditional strategies in terms of specific productivity (33-34).

Dissolved oxygen is a key fermentation parameter; especially for *P. pastoris* growing on methanol were the demand is too high for the degradation of methanol to formaldehyde by alcoholoxidase into the peroxisome (35-36). Standard protocols fix the set-point of dissolved oxygen higher than 20% trying to avoid hypoxic conditions.

The importance of these parameters has also been shown in bioprocess under P_{GAP} (37). It is well known that the effect of specific growth rate (μ) has an important effect on the bioprocess productivity independently of the operational mode selected (38-39) and classical approach to get the objective to maintain the specific growth rate at the optimum setup are well known (40-41). In Erlenmeyers had been observed that significant improvements in the protein production parameters were reached upon short time depletion of substrate (glucose) (42). From these previous results, high-cell density fed-batch cultures at high specific growth

rate were made to study the effect of carbon source starvation periods on the secretory efficiency of the cell factory and the effect on productivity and yields. The strategy was applied to the production of both light and heavy chain genes on the humans Fab2F5. The application of carbon-starving periods in a classical exponential feeding rate maintaining a constant specific growth rate in open-loop control scheme increases up to 50% of yields and total production. Thus, this non-complex cultivation strategy is a significant operational improvement for P_{GAP} based expression systems (43).

Studies of physiological and metabolic adaptation of *P. pastoris* under GAP promoter using glucose as substrate in a wide range of oxygen availability has demonstrated an increase of protein production as a consequence of the metabolic shift from respiratory to respiro-fermentative pathways open a new approach to exploit the positive effects of oxygen-limiting conditions in protein expression (44)

In the last five years, all this tools have applied to improve the production or recombinant proteins in general and recombinant lipases in particular in the cell factory *P. pastoris*.

Novel results obtained for the heterologous production of three of the more used lipases in biocatalysis and new cell factories are presented in the next sections.

RECOMBINANT *Candida antarctica* lipase B (CALB)

Recently *Candida antarctica* lipase B (CALB), one of the most applied lipase in biotransformations, has been expressed under alternative promoters. A novel synthetic P_{AOX1} variant has been characterized physiologically to demonstrate its functionality and to study the dynamic conditions for switching in order to control these modified promoters using appropriate process strategy (31). CALB has also been successfully expressed under the constitutive PGK1 promoter. This promoter is related to the glycolytic enzyme 3-

phosphoglycerate kinase (45). PGK1 promoter was as efficient as the classical GAP promoter (46).

The effect of overexpression of molecular chaperones in *P. pastoris* to increase the production of CALB has also proved. Hsp70 and Hsp40 chaperone families regulate the folding and secretion of heterologous protein. Different chaperones (Ydj1p, Ssa1p, Sec63p and Kar2) were tested in individual overexpression or in combination. The maximum increase in the expression level was 2.5-fold with the combination Ydj1p-SSa1p. On the other hand, the overeexpression of Kar2p resulted in a decrease of CALB production, showing that this chaperone is not required for the secretion of CALB (47).

The optimization of the codons of both CALB gene and α factor signal peptide using a *de novo* design and synthesis strategy let to obtain a high efficient expression (48).

CALB has also been expressed as fusion on the cell wall in anchored glycoproteins of *P. pastoris* (49).

From a bioprocess engineering pint of view, the effect of initial crude glycerol concentration from biodiesel production has been tested as carbon source in batch cultures for the recombinant production of CALB under the constitutive new PGK1promoter. The maximum $Y_{P/X}$ and $Y_{P/S}$ were obtained for a 100 g L^{-1} of initial crude glycerol concentration. However, in terms of specific productivity the maximum value was reached at the lower crude glycerol concentration. A simple strategy to implement the addition of the initial crude glycerol concentration in four pulses of 25 g L^{-1} during the exponential growth rate phase increased the production of CALB 1.5 fold times (46).

CALB has successfully expressed into the *Pichia* expression plasmid pPICZαA with a classical approach of methanol feeding rate (50).

Bioprocess engineering of CALB has also investigated with a two gen copies strain coexpressing the protein disulphide isomerase (PDI) under the inducible AOX1 variant promoter. The temperature of the cultivation was 25ºC lower than the standard of 30ºC. The relationship between specific CALB secretion rate and specific growth rate was stablished for *P. pastoris* growing on glycerol under limited substrate availability (< 1 g L^{-1}) in fed-batch cultures. With this new approach from synthetic biology and bioprocess engineering, the results suggesting that the secretion kinetics observed are not determined by promoter regulation but instead by production/secretion capacity (31).

RECOMBINANT *Rizhopus oryzae* lipase (ROL)

The increase gene dosage is a classical approach to increase productivities in heterologous protein production. However in heterologous ROL production this strategy at high-copy strains reduced cell growth, ROL production and substrate consumption (12).

The results obtained from transcriptomics analysis in a subset of strain from 1-15 ROL copies confirmed that growth yield and carbon uptake rate were gene dosage dependent. The increased number of cassettes led to transcription attenuation of the methanol metabolism and peroxisome biogenesis, jointly with a reduction in secretion levels. This kind of studies let a better asses of physiological response of the microorganisms (Cámara et al., 2017).

The use of *omics* has also applied in ROL production. Glucose-methanol and glycerol-methanol co utilization in *P. pastoris* studies by metabolomics and instationary ^{13C}fux analysis has been made with ROL under AOX1 promoter (36,52-54). This approach has provided a valuable tool for the metabolic characterisation of *P pastoris* when a mixed substrate has been applied, assessing the impact of recombinant protein production on central carbon metabolism when glucose or glycerol is used as (co)substrate (36,52).

These previous result of ROL metabolomics continued with the study of time profile of free amino acid and central carbon metabolism intermediates in glucose limited chemostat cultures (55). The quantitative metabolomics analysis of amino acid metabolism under different oxygen availability conditions combined with previous transcriptomics and proteomic analyses provided a systematic metabolic fingerprint of oxygen availability impact on recombinant ROL production in *P. pastoris* (56).

Under inducible promoters as P_{AOX1} different operational strategies has been tested for the heterologous production or *Rhizopus oryzae* lipase (ROL) with the objective of searching the best operational strategy for a Mut^+ phenotype. Surprisingly, in methanol limited fed-batch cultures (MLFB), all production parameters were very low. Same results were obtained for methanol non limited fed-batch cultures (MNLFB) for a methanol-set-point lower than 2 g L^{-1}. However, at higher methanol set-point a significant increase in the production was observed. Optimum methanol set-point was reached at 3 g L^{-1}. Inhibitory effect of ethanol on cell growth and ROL production was observed at methanol concentration of 10 g L^{-1} (57). All this information let the proposal of an unstructured macrokinetic model for ROL under this promoter. The model using a non- monotonical substrate function for growth, Pirt's model for substrate uptake and Luedekin-Piret equation for protein production let the overall validation for the entire operational mode (30).

Quite interesting is a comparative analysis of different target protein production under this promoter obtaining variable ranges of yields and specific rates (30).

Recently a study to know if ROL production is oxygen tension dependent has been made (59). In terms of total ROL productivity a set-point of 25% was the optimum and confirming that low set-point values (5%) diminish this values 4 fold. However, the highest overall product yield was obtained at a set-point of 10%. Finally, the study confirms that the specific rates and

$Y_{P/X}$ yield were lower at the extremes of the DO range tested (59). Thus, this parameter has to be controlled in order to maximize the production but also to get a reproducible bioprocess.

The effect of mixed substrates feeding (methanol-glycerol) and dilution rate in chemostat cultures has also been tested in the production of ROL under AOX1 promoter. The results demonstrated that ROL production is strongly dependent of dilution rate and an increase in the volumetric productivity with glycerol-methanol feeding was higher than with methanol feeding as sole carbon source. The optimum dilution rate was reached at high values 0.04-0.06 h^{-1} with ROL yield decreased a lower dilution rates. A kinetic analysis showed that the obtained results are justified by the effects of dilution rate on specific methanol uptake rate, instead of the well-known repressor effect of glycerol on P_{AOX1} (60).

Other associated problem is proteolytic degradation. A set of operational strategies including medium composition, pre-induction osmotic stress and temperature has been applied to minimize this problem in recombinant ROL production (49).

The pro-form lipase from *R. oryzae* has also been functionally expressed under AOX1 promoter under classical and non-optimized high cell density fed-batch cultivation in a 50-L bioreactor (61). This pro-form is involved in the correct folding and efficient secretion of mROL getting a more stable biocatalyst (62).

The performance of ROL onto the cells *of P. pastoris* using Sed1p as an anchor protein has been studied. The displaying lipase was stable in broad ranges of temperatures and pH. Thus, *P. pastoris* displaying lipase may have potential as whole-cell biocatalyst (21).

3.- RECOMBINANT *Candida rugosa* LIPASE (CRL)

The isoenzyme LIP1 of *C. rugosa* has been overexpressed in an engineered *P. pastoris* strain, under AOX1 promoter, including a set of improvement genetic previously commented. The

LIP1 gene was systematically codon optimized and synthesized *in vitro*, the effect of a multicopy strains was tested on LIP1 production. Finally co-expressing of one copy of ERO1p and BIP chaperones were also included in the strain. The coexpression of the two chaperones increased in 46,5% the enzyme yields.

Non-conventional fed-batch cultivation strategy was also applied. In the methanol induction phase, the temperature of the bioprocess was reduced to 27ºC, and the effect of mixed substrates (sorbitol) were tested. The study demonstrates that this approach combining upstream genetic operations and downstream fermentation optimization has a great potential for the industrial-scale production of LIP1 (63).

The isoenzyme LIP2 of *C. rugose*, probably the most difficult isoenzyme to express of the eight *LIP* genes (*LIP1* to *LIP*8) (64-65) was get under constitutive GAP promoter. The combination of a multicopy strain with low-temperature culture resulted in a maximal 32-fold increase in LIP2 secretion (66). A linear correlation between gene copy number and lipase activity was reached up to three copy number. For a four copy number no increase in lipase activity was observed. The mRNA amount was positively correlated with the increase in copy number up to three copies. The authors pointed out that not only the secretion pathway but also the steady-state mRNA transcript amount determines the expression level of extracellular protein (66).

NEW CELL FACTORIES PRODUCING RECOMBINANT LIPASES

Trichoderma reesei, has emerged as other potential cell factory for the production of heterologous lipases. *Aspergillus niger* lipase has been *de novo* synthesized and expressed in *T. reesei* under the promoter of the cellobiohydrolase 1 gene (*cbh1*). The production was increased up to 3.2 fold by RNAi-mediated gene silencing via downregulation of CBHI

responsible of more than 60% of the total extracellular protein in *T. reesei.* This strategy helps to reduce specific gene expression in *T. reesei* which has a very low homologous recombination rate (67).

Talaromyces thermophilus lipase gene has also been successfully expressed in *T. reesei* using *cbh1* promoter. The big advantage of this fungal lipase is the high stability at both alkaline conditions and high temperature. Thus, this heterologous lipase is very promising for industrial applications like paper making and detergent addition (68). On the other hand the synergetic degradation of cellulose and lipase makes this modified microorganism an excellent candidate to the treatment of oil-rich mill waste treatment, where the cellulose and triglycerides coexist (69-70).

The yeast *Candida utilis* has been successfully used as a cell factory for the heterologous production of CALB. The major advantage of *C. utilis* compared to other established production hosts including *Pichia* or *Hansenula* yeasts lies in its robust growth properties, in particular its ability to grow on xylose and other inexpensive carbon and nitrogen sources, as well as its tolerance to elevated temperatures and extremes in pH. In combination with its ability to secrete proteins to the growth medium or to place/fix proteins to the cell wall, these properties make *C. utilis* a promising host for biotechnology applications (71).

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