Microarray-Based Transcriptome Analysis of Recombinant *Pichia Pastoris* Strains Overexpressing Alpha-Amylase and Interleukin-2

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Abstract — **DNA microarray has been a useful tool for global-scale transcriptome analysis. To study the cellular response to expression of recombinant proteins, we compared the transcriptional profiles of recombinant *Pichia pastoris* strains overexpressing amylase and interleukin-2 versus that of the control strain at different cellular states. The microarray analysis was carried out via the use of Yeast_2 array specific for *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The transcirptome analysis of each studied strain at logarithmic growth phase and stationary growth phase showed hundreds of significant differences. In contrast, in comparison of studied strains at the same time points, the numbers of gene which are differentially expressed is rather low. Interestingly, the expression of heterologous alpha-factor secretion signal in the strains overexpressing amylase and interleukin-2 was up-regulated by more than 15 times and 140 times at the exponential and stationary phase, respectively. The results also provide evidence about the false positive result in microarray data when using non-specific array.**

Index Terms—Pichia pastoris, **microarray, transcriptome, IL-2, α-amylase**

I. Introduction

The methylotrophic yeast *Pichia pastoris* is a suitable host for production of heterologous proteins [1], [2]. It is observed that some proteins are produced at high level whereas other proteins are secreted at low yield in this system. The expression of foreign proteins can trigger the host cell response and then make changes to the process of cell metabolism. These changes can lead to instability of the foreign gene, disruption of ribosome structure, inhibition of the growth or even the destroy of the cell. Thus, these may adversely affect the ability to generate recombinant protein. To solve this problem, a number of studies have attempted to improve the recombinant proteins expression by the alteration of the genetic codes [3], co-expression along with a chaperone or with other proteins [4], [5], optimization of culture conditions [6], [7] and modification of genetics of the host strain [8]. The information established from the approaches can also be applied to increase production of other recombinant proteins, but not all cases are successful as

basic knowledge of the yeast physiology and molecular genetics is still not sufficient for a comprehensive assessment of the heterologous gene expression.

DNA microarray is a powerful tool to study transcriptional expression at the global scale. In *P. pastoris,* microarray-based transcriptome analysis has been applied to elucidate the regulation of host cell during protein production. Most of the researches have mainly focused on analyzing different transcriptional expression patterns of the recombinant strain under stress conditions such as temperature, oxygen, etc for understanding the molecular mechanism of the heterologous gene expression [9]-[11]. The result of the transcriptome analysis can be used for enhancing secretion of heterologous protein in the yeast [12].

Whole genome sequences of two *P. pastoris* strains GS115 and DSMZ 70382 have been published [13]-[15]. Nevertheless, the array specific for *P. pastoris* has not yet been available. Due to that fact, most of global transcriptional studies in *P. pastoris* so far have been conducted via the use of arrays from closely related yeast species e.g. *S. cerevisiae.*

In our study, the transcriptomes of the recombinant *P. pastoris* strains producing alpha-amylase, interleukin-2 were investigated in methanol induced cultures at different time points. The main aim of this work is to monitor the transcriptional patterns of the recombinant *P. pastoris* strains harboring different genes to obtain valuable information for understanding the gene expression and then propose a suitable way to improve the recombinant protein expression. The transcriptional profiles of the studied strains were analyzed via the use of Yeast_2 microarray specific for *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.

II. Materials and Methods

A. Strains Used in the Study

Strains used in this study include two recombinant *P. pastoris* strains overexpressing α-amylase and interleukin-2 and their relevant control strain. For simplicity, the strains were respectively denoted as Amy, IL-2 and control strains. The control strain was generated via the integration of pPIC9 vector into the host *P. pastoris* SMD1168 genome at *his4* locus. Accordingly, the integration of pPIC9 vector harbouring recombinant gene (either *AMY or IL-2)* into the SMD1168 genome results in the generation of the recombinant yeast strains. The recombinant gene encoding α-amylase was derived from *S. fibuligera* while the recombinant *IL-2* was originated from human.
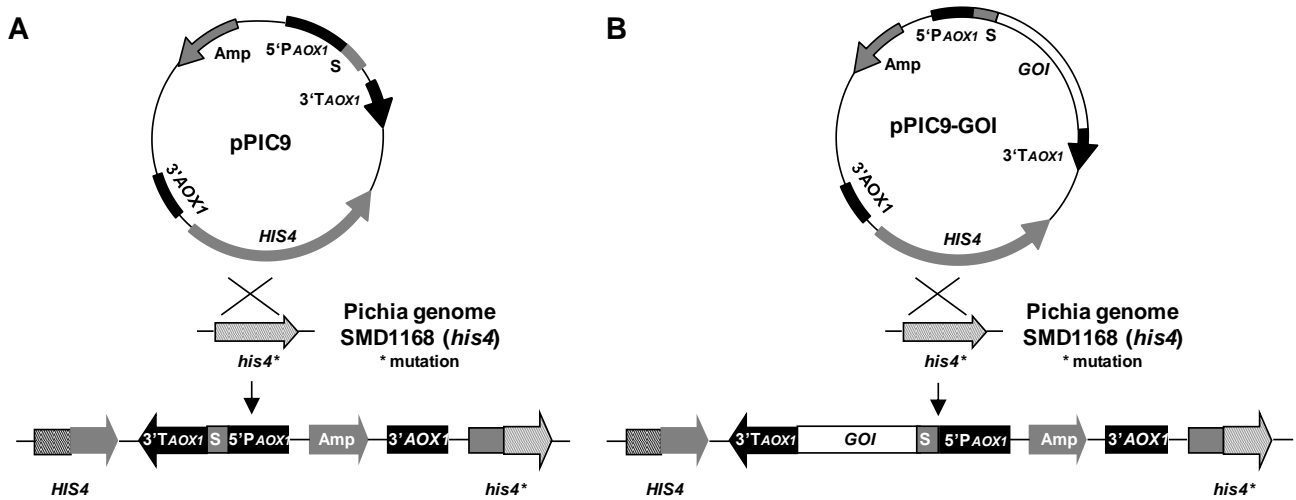
Fig. 1. Strategy employed for generation of studied *Pichia pastoris* strains. A: control strain; B: Strains overexpressing recombinant proteins, i.e. amylase (AMY) or interleukin-2 (IL-2). 5'P$_{AOX1}$: 5' *AOX1* promoter fragment; S: α-factor secretion signal (*MF(ALPHA1)*); 3'T$_{AOX1}$: 3' *AOX1* terminator; 3'*AOX1*: 3' *AOX1* fragment; Amp: Ampiciline resistance gene; GOI: gene of interest

B. Fermentations

Yeast cells from MD agar plates were pre-cultured into 100 ml Erlenmeyer flasks containing 20 ml of BMGY. The precultures were incubated at 28 °C on a rotary shaker (250 rpm) overnight until optical cell densities (OD$_{600}$) of 6 were reached. The precultures were inoculated into 1 litre Erlenmeyer flask containing 300 ml BMGY [16] at OD$_{600}$ of 0.1 for fermentation with shaking at 250 rpm at 28 °C. When the cell optical density (OD$_{600}$) of fermentation reached to the values from 15 to 16, the fermentation cultures were induced with MeOH at the final concentration of 1%. After that, the cultures were induced with MeOH (1%) twice a day during the whole fermentation.

C. Microarray-Based Comparative Transcriptome Analysis

For RNA isolation, cells were harvested from 1 litre flask fermentation at 0 h before the first MeOH induction and at 24 h and 48 h after the first MeOH induction. About 4×10^7 cells were separated from fermentation broth via centrifuging at 5000 rpm (Biofuge Fresco Heraeus, rotor 3325B) at 4 °C. Cells were then resuspended in 2 ml GITC-containing lysis buffer (RLT buffer from RNeasy Mini Kit, Quiagen [17]) and fast frozen in liquid nitrogen. The frozen cells were kept at -70 °C for about 1-3 weeks before being shipped on dry ice to Asuragen Inc./ Texas for further analysis.

RNA isolation was done by Asuragen Inc. following the RiboPureTM-Yeast Instruction Manual (http://www.ambion. com/techlib/prot/fm_1926.pdf). Afterwards, microarray analyses were performed using Affymetrix® Yeast Genome 2.0 Gene Chips following the standard protocol (http://www.affymetrix.com/support/technical/manual/expre ssion_manual.affx). With standard processing, 2 μg were used for preparation of biotin-labelled targets (cRNA) using modified MessageAmp™ -based protocols (Ambion Inc., Austin, TX). The cRNA yields were quantified by spectrophotometry and the distribution of transcript sizes was assessed using the Agilent Bioanalyzer 2100 capillary electrophoresis system. Labelled cRNA was fragmented in a 0.5 μg/μL reaction and used for array hybridization and washing, according to the standard Affymetrix protocol. In brief, labelled cRNA was resuspended in 5X fragmentation buffer and incubated at 94 °C for 35 minutes then stored on ice. The hybridization cocktail and the fragmented cRNA mixture were heated to 99 °C for 5 minutes, and incubated at 45 °C for 5 minutes. After a final spin to collect the samples, hybridization to arrays was carried out at 45 °C for 16 hours in an Affymetrix Model 640 hybridization oven. Arrays were washed and stained on an Affymetrix FS450 Fluidics station. The arrays were scanned on an Affymetrix GeneChip Scanner 3000 7G. A summary of the image signal data, detection calls, and gene annotations for every gene interrogated on the array was generated using the Affymetrix Statistical Algorithm MAS 5.0 (GCOS v1.3) algorithm, with all arrays scaled to 1500. The Affymetrix data were reported in a .dtt (data transfer tool) file containing the Affymetrix data and result files.

For statistical analysis, at first null hypothesis was tested with two-way ANOVA (Analysis of Variance) in multiple group comparison. The goal of this test was to filter out genes that have same expression level across all groups. After that, pair-wise comparison was performed for all interactions to identify significant differences. For every transcript in the pair-wise comparison, an average logged fold-change was calculated. For each pair of comparison, two-sample t-test was carried out for every gene and the derived p-values were adjusted for multiple testing and converted to false discovery rate (FDR) applying Benjamin and Hochberg procedure. As significant threshold for the pair-wise comparison, a false discovery rate of 0.05 was chosen.

D. SDS-PAGE Analysis and Protein Band Evaluation

The fermentation culture was centrifuged at 5000 rpm (Biofuge Fresco Heraeus, rotor 3325B) for 5 min at 4 °C. The extracellular protein was treated in denatured buffer at 95 °C for 5 min and SDS-PAGE was performed according to protocol of Laemmli [18]. The SDS-PAGE gels were stained with silver for visualization [19] and with Comassie Blue for band size evaluation. The band size analysis was carried out using Quantity one® version 4.6.3 software (Bio-Rad, Hercules, CA.

III. Results

To study the cellular response to the expression of recombinant proteins in the host *P. pastoris*, we performed

microarray-based transcriptome analysis of *P. pastoris* strain SMD1168 (control) and the two recombinant SMD1168 strains overexpressing α-amylase (Amy) and interleukin-2 (IL-2). The yeast transcriptional profiles were analyzed 0 h before the first MeOH induction and at 24 h and 48 h after the first MeOH induction. For simplicity, the points of time when the yeast cells were harvested are denoted as 0 h, 24 h and 48 h. At the studied fermentation conditions, all strains showed the similar growth curve (data not shown). At 0 h, all yeast strains were in the logarithmic growth phase of which cell optical densities OD_{600} varied from 15 to 16. At 24 h and 48 h, cells were at the stationary phase and showed similar cell optical densities OD_{600} of about 30. At the points of time when the cells were harvested for RNA isolation, we also collected the fermentation broth to examine secretion of extracellular protein.

A. Extracellular Protein Secretion in Recombinant Strains

The extracellular protein secretion in recombinant yeast strains was examined on Acrylamid gel (Fig. 2). The recombinant proteins IL-2 and amylase were not detected at 0 h. The abundances of each recombinant protein (IL-2 and amylase) at 24 h and 48 h were quite similar. In addition, analysis of band size using Quantity one® version 4.6.3 software on coomassie *stained* gel shown that the amylase bands at both 24 h and 48 h were about 4 times higher than those of IL-2 bands (data not showed). It can be explained by the fact that the MW of amylase is 55.0 kDa, about 4 times higher than that of Interleukin-2 (14.0 kDa). When comparing both band size and molecular weight of Amylase and IL-2, we concluded that, at the studied conditions, the two *P. pastoris* strains overexpressing Amylase and IL-2 showed the same level of recombinant protein expression.

B. Microarray Based-Transcriptome Analysis

The experiment was carried out in triplicate in which cell samples used for RNA isolation were harvested from three independent fermentations. In total, for three strains studied at three time points of three fermentations, 27 samples were analysed. The hybridisation signals generated for each strain at each time point were *calculated* as the mean value from three different fermentations. Afterwards, pairwise transcriptomic comparison was carried out according to time variable (one strain at different time points of the fermentation) and strain variable (different strains at one similar time point of the fermentations).

In microarray-based transcriptome analysis, we used Affymetrix GeneChip®Yeast Genome 2.0 Array [20]. This array contains probe sets to detect transcripts from both *S. cerevisiae* and *S. pombe*, the two most commonly studied species of yeast. The array includes probe sets to detect 5,841 genes in S. *cerevisiae* and 5,031 genes present in *S. pombe*. Every probe set contains 11 probes. Each probe is an oligonucleotide of 25 bp long, hybridized to the target sequence.
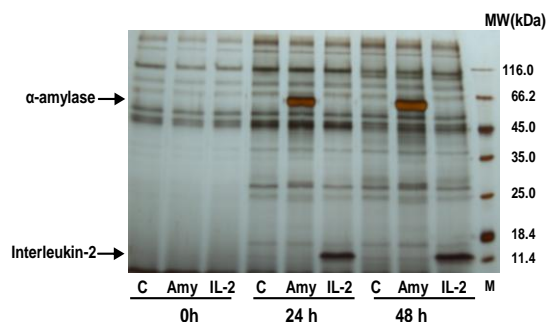

Fig. 2. Analysis of extracellular proteins from recombinant strains produced during fermentation. Crude extracellular protein extracts from culture supernatant of recombinant strains were separated on SDS-PAGE in 12.5 % gels. Protein detection was done by staining with silver nitrate. C: control strain; Amy and IL-2: recombinant strains overexpressing α-amylase and interleukin-2, respectively.
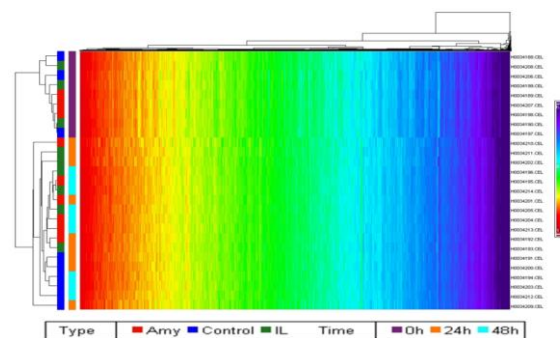

Fig. 3. Heat map of differentially expressed transcripts transcriptome analysis of three studied strains at different time points of fermentations 0 h, 24 h and 48 h.

The transcriptional analysis of three strains according to time variable *showed* hundreds of genes differentially expressed in the comparison at 0 h versus those at 24 h and 48 h (TABLE I). In contrast, no significant difference was detected in the transcriptome comparison between 24 h and 48 h.

To study the cellular mechanism responding to the expression of recombinant proteins, we compare the transcriptional profiles of three studied strains at the several points of time during the fermentation. According to strain variable, only 6 significant differences were identified at the studied time points of 0 h, 24 h and 48 h (TABLE II). These 6 differences are detected in the comparison between the control strain and either with the IL-2 or Amy strains. No significant difference was detected in the analysis between IL-2 and Amy strains. Among those, it is remarkably that *MF(ALPHA)1* gene is up-regulated by more than 15 times at 0 h in the recombinant strains Amy and IL-2 compared to the control strain. After the first methanol induction, *MF(ALPHA)1* transcript about more than 140 times higher in the recombinant strains at both 24 h and 48 h comparing to the control strain at the same time points (TABLE II, TABLE III). The difference regarding *MF(ALPHA)1* transcript abundance is both identified in comparison regarding time and strain variables of the studied strains.

TABLE I: Number of significant differences identified at transcriptional level of the studied P. pastoris strains according to time variable. For each pairwise comparison, the number of significant differentially expressed genes was considered with a false discovery rate of 0.05.

No of significant differences (Time)	Control			Amy			IL-2		
	24h/0h	48h/0h	48h/24h	24h/0h	48h/0h	48h/24h	24h/0h	48h/0h	48h/24h
Total	433	351	0	645	447	0	542	435	0
Increased	211	170		282	210		273	200	
Decreased	222	181		363	237		269	235	

TABLE II: SIGNIFICANT DIFFERENCES IDENTIFIED AT TRANSCRIPTIONAL LEVEL OF THE STUDIED STRAINS ACCORDING TO STRAIN VARIABLE. FOR EACH PAIRWISE COMPARISON, THE NUMBER OF SIGNIFICANTLY DIFFERENTIALLY EXPRESSED GENES WAS CONSIDERED WITH A FALSE DISCOVERY RATE OF 0.05. GREY-MARKED SHEETS INDICATE SIGNIFICANT DIFFERENCES IDENTIFIED IN A PAIRWISE COMPARISON ACCORDING TO VARIABLE OF STRAIN.

Gene	Probe ID	Hybridization ratios								
		0 h			24 h			48 h		
		Amy/ Control	IL-2/ Control	Amy/ IL-2	Amy/ Control	IL-2/ Control	Amy/ IL-2	Amy/ control	IL-2/ Control	Amy/ IL-2
MF(ALPHA)1	1775057_at	15.6	19.3	0.8	146.6	153.4	1	159.0	161.0	1.0
Bla	RPTR-Sc-J002682-2_s_at	24.8	39.7	0.6	20.0	20.0	1.0	15.2	19.1	0.8
rpl1001	1769984_at	1.3	1.6	0.8	5.0	4.3	1.2	3.5	3.8	0.9
GAL2	1773096_at	0.8	0.9	0.8	0.9	0.9	1.0	1.0	1.0	1.1
RPB2	1772025_at	1.4	1.0	1.4	0.9	0.9	1.1	0.9	1.0	1.0
PTK2	1772327_at	1.0	1.0	1.0	1.0	1.2	0.9	1.1	1.5	0.8

*) Note: *MF(ALPHA)1*: Mating pheromone alpha-factor, made by alpha cells; interacts with mating type a; *Bla*: control gene, encoding antibiotics, produced by some bacteria; *Rpl1001*: 60S ribosomal protein L10; *GAL2*: Galactose permease, required for utilization of galactose; also able to transport; *RPB2*: RNA polymerase II second largest subunit B150, *PTK2*: Putative serine/threonine protein kinase involved in regulation of ion transport.

TABLE III: AVERAGE HYBRIDIZATION SIGNALS OF 6 SIGNIFICANT DIFFERENCES IDENTIFIED IN THE TRANSCRIPTIONAL ANALYSIS ACCORDING TO STRAIN VARIABLE. ANNOTATION FOR GENES AND RELEVANT PROTEINS IS SHOWN IN TABLE 2. THE SIGNALS OF EACH GENE WERE CALCULATED AS THE MEAN VALUE FROM THREE INDEPENDENT HYBRIDIZATIONS.

Gene	Probeset ID	Mean Control 0 h	Mean Control 24 h	Mean Control 48 h	Mean Amy 0 h	Mean Amy 24 h	Mean Amy 48 h	Mean IL-2 0 h	Mean IL-2 24 h	Mean IL-2 48 h
MF(ALPHA)1	1775057_at	76.1	98.6	84.4	1187.2	14453.2	13419.1	1471.9	15118.2	13584.7
Bla	RPTR-Sc-AJ002682-2_s_at	37.5	25.3	35.4	929.4	505.5	539.8	1486.2	500.0	677.0
rpl1001	1769984_at	652.6	249.4	324.7	847.0	1254.6	1126.8	1054.4	1073.6	1240.7
GAL2	1773096_at	111.2	117.4	110.4	83.9	104.4	114.9	102.7	107.0	104.7
RPB2	1772025_at	14.7	13.5	13.9	21.0	12.5	13.1	15.3	11.9	13.4
PTK2	1772327_at	7.6	7.2	6.1	7.9	7.6	7.0	7.5	8.3	8.9

TABLE IV: DNA SEQUENCE COMPARISON OF SEQUENCES ENCODING 60 RIBOSOMAL PROTEINS OF S. CEREVISIAE AND S. POMBE VERSUS THAT OF P. PASTORIS GS115 AND DSMZ 70382. IN S. POMBE, THE SEQUENCE IS DENOTED UNDER LOWERCASE LETTER WHILE IN S. CEREVISIAE AND P. PASTORIS, THE GENES ARE NAMED IN CAPITAL LETTERS.

YEAST_2 probe sets	Probet set target sequence	Similarity to *P. pastoris RPL10* sequences	
		GS115	DSMZ 70382
1769984_AT	rpl1001, encoding 60S ribosomal protein L10 from *S .pombe*	69%	69%
1775248_S_AT	rpl1001, encoding *S. pombe* 60S ribosomal protein L10	69%	69%
	rpl1002, encoding *S. pombe* 60S ribosomal protein L10		
1773845_AT	rpl1002, encoding *S. pombe* 60S ribosomal protein L10	65%	65%
1770948_AT	RPL10, encoding *S. cerevisiae* protein component of the large (60S) ribosomal subunit, responsible for joining the 40S and 60S subunits; regulates translation initiation;	81%	80%

IV. Discussion

The changes in transcriptional profiles of yeast strains at different cellular states have been previously reported in various studies [21], [22]. In our experiment, the identified significant differences according to time variable are absolutely consistent to the physiology of the studied yeast strains. Concretely, at 0 h, the studied strains were at exponentially growth while at 24 h and 48 h; they were already in stationary phase. Due to that fact, the transcriptional profiles of all studied yeast strains at 0 h are very much different from those at 24 h and 48 h while no change between 24 h and 48 h was detected. In addition to reason of cellular state metabolism, it should not be excluded that the methanol induction to studied yeast strains after 0 h is a factor responsible for differential transcriptional expression. As the yeast cells were induced with methanol at 0 h, the sequences under *AOX1* promoter including alpha secretion signal, recombinant genes and native *AOX1* genes were overexpressed, contributing to the transcriptional differences between 0 h versus 24 h and 48 h.

As our goal in this study is to investigate the cellular response to the expression of recombinant proteins, our analysis does focus on the transcriptional changes at cellular states but rather on the changes occurring among three studied strains at the same points of time.

In the paiwise comparison according to strain variable, the expression of *GAL2* and *RPB2* genes are showed to be slightly dissimilar in the control and Amy strains at 0 h (TABLE II). The fold-change of difference was not high and at this time point, expression of recombinant amylase has not yet been induced. We therefore assumed that these differences regarding *GAL2* and *RPB2* genes may not be really related recombinant protein expression.

In our analysis, it was showed that expression of rpl10 gene encoding for 60S ribosomal protein L10 was not much different at 0 h in all three studied strains (TABLE II). However, at 24 h and 48 h, this gene was up regulated more than 3.5 times in strain IL-2 and Amy. Theoretically, this result can be explained that as transcription of recombinant genes is regulated under the *AOX1* promoter, thus, after the 1st methanol induction, recombinant genes was overexpressed and recombinant proteins were accumulated. This in turn might result in the overexpression of ribosomal protein for extracellular transportation. Nevertheless, by the time when our array experiment was completed, the genome of *P. pastoris* strains GS115 and DSMZ 70382 have been sequenced and published [13]-[15]. We therefore referred to these two genome databases to further study the difference regarding rpl10 gene expression. The reference of *P. pastoris* genome database has led to the unexpected conclusion on our result.

S. cerevisiae and *S. pombe* respectively contain 1 and 3 genes encoding ribosomal proteins (TABLE IV). The Yeas_2 array contains 4 different probe sets to detect these 4 genes. Among those 4 probe sets, only the probe set 1769984_AT (specific for rpl10 gene in *S. pombe*) resulted in different hybridization signals in microarray-based transcriptome comparison according to strain variable while other probe sets did not. It is showed that the sequences of *RPL10* in *P. pastoris* GS115 and DSMZ 70382 strains are 97% identical. However, the blast of all probes of 1769984_AT probe set against genomes of *P. pastoris* strains GS115 and DSMZ 70382 revealed that these probes have low similarity not only with *RPL10* but also with other *ORFs*. In other words, the probe set 1769984_AT was not specific enough for studying *P. pastoris* transcriptional expression. The obtained difference regarding this probe set may result from unspecific and/or cross-hybridization of the probes to other transcripts of studied *P. pastoris* strains.

Similarly, profound analysis on *PTK2* sequence of *P. pastoris* also revealed that the significant difference regarding *PTK2* gene was not reliable (data not showed). *PTK2* sequence from *P. pastoris* was much different from those from *S. cerevisiae* and *S. pombe (*only about 45% identical). Our blast also revealed that the relevant probe sets used to detect *PTK2* from *S.cerevisiae* and *S.pombe* was also not specific for studying *P. pastoris* transcriptional expression. In addition, the hybridization signal generating from 1772327_at probe set responsible for *PTK2* sequence is very low (TABLE III), suggesting that the difference detected by this probe set was due to noise from background signal.

The abundance of *MF(ALPHA)1* transcript has been identified as a notable significant difference in transcriptome analysis of studied strains. In fact, the reference of native *P. pastoris* genomes showed that there is no *MF(ALPHA)1* in this host. Nevertheless, the genomes of the studied recombinant *P. pastoris* strains contain a part of the *MF(ALPHA)1 (*270 bps*)* from *S. cerevisiae* which was introduced into the integration cassette to serve as the α-factor secretion signal (Fig. 1). As this part is originated from *S. cerevisiae*, the relevant probe set from Yeast_2 array is specific for the detection of this part of *MF(ALPHA)1* gene in the studied *P. pastoris* strains. The α-factor secretion signal and the recombinant gene are under the same *AOX1* promoter (Fig. 1). However, in the control strain where there is no recombinant gene, the expression of α-factor secretion signal was not changed after MeOH induction (TABLE III). In contrast, in the *P. pastoris* strains overexpressing amylase and IL-2, the α-factor secretion signal was already increased by more than 15 times at 0 h comparing to control strain, suggesting that transcription of recombinant genes and α-factor secretion signal was already initiated before MeOH induction. After MeOH induction, the expression of this α-factor secretion signal is further increased at high level about 140 times in the recombinant strains comparing to control strain (TABLE III). The expression level of *MF(ALPHA1)/*alpha secretion signal seems to be affected by the expression of recombinant genes amylase and interleukin-2. It is assumed that alpha secretion signal was synthesized in the control strain at lower level as this strain did not have the demand for protein secretion as the two recombinant strains. Another hypothesis is α-factor secretion signal was transcribed at the same level in the all studied strains. However, in the control strain, the α-factor secretion signal was degraded as the control cells do not need this signal for protein secretion.

Bla gene encoding for beta-lactamase is a control gene from bacteria and not present in the native host *P. pastoris* strains. However, the *bla* gene is in fact the Amp gene in the integration cassette of our three recombinant *P. pastoris* strains. Amp/bla gene is not designed to be regulated by *AOX1* promoter. The microarray analysis showed that compared to the control strain, bla gene is about more than 15

times up-regulated in Amy and IL-2 strains at all studied points of time 0 h, 24 h and 48 h (TABLE II, TABLE III). We so far have no suitable explanation for this result.

V. CONCLUSIONS

The microarray analysis of *P. pastoris* strains overexpressing recombinant protein interleukin-2 and amylase via the use of Yeast_2 array revealed some significant differences relevant to expression of alpha secretion signal and marker gene bla gene. No cellular response relevant to expression of native genes from *P. pastoris* was detected in the pairwise transcriptional comparison regarding strain variable.

The profound analysis on some identified significant differences using recently *P. pastoris* genome sequence has also revealed that some identified significant different in fact was not reliable as the probe sets designing to detect *S. cerevisiae* and *S. pombe* from the Yeast_2 array used for this experiment was not specific enough to detect the relevant *P. patoris* genes. Regarding this fact, it could not be ensured that the transcriptional profile studied *P. pastoris* yeast strains really showed no other significant differences besides α-factor secretion and bla gene. In our experiment, it can happen the case that, the integration of the studied recombinant genes indeed results in up/down regulation of certain genes. Nevertheless, these significant differences were not reflected via the probet sets of Yeast_2 array. To have a comprehensive understanding on the cellular response to the expression of recombinant genes in host *P. pastoris*, a specific array is required. The results also stress that precaution should be taken when we evaluate transcriptional data using non-specific microarray.

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