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Combined use of *GAP* and *AOX1* promoters and optimization of culture conditions to enhance expression of *Rhizomucor miehei* lipase

Dong He[1,2] · Wen Luo[1] · Zhiyuan Wang[1] · Pengmei Lv[1] · Zhenhong Yuan[1]

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Abstract *Rhizomucor miehei* lipase (RML) is an industrially important enzyme, but its application is limited due to its high cost. In this study, a series of measures such as codon optimization, propeptide addition, combined use of *GAP* and *AOX1* promoters, and optimization of culture conditions were employed to increase the expression of RML. Three transformants of the constitutive-inducible combined *Pichia pastoris* strains were generated by transforming the pGAPZαA-*rml* vector into the pPIC9K-*rml*/GS115 strain, which resulted in high-expression yields of RML. Using the shake flask method, highest enzyme activity corresponding to 140 U/mL was observed in the strain 3-17, which was about sixfold higher than that of pPIC9K-*rml*/GS115 or pGAPZαA-*rml*/GS115. After optimization of culture conditions by response surface methodology, the lipolytic activity of strain 3-17 reached 175 U/mL in shake flasks. An increase in the copy number simultaneously with the synergistic effect provided by two promoters led to enhanced degree of protein expression.

Keywords RML · *Pichia pastoris* · P_{GAP} · P_{AOX1} · Optimization

Introduction

The extracellular lipase enzyme (triacylglycerol ester hydrolase, EC 3.1.1.3) from *Rhizomucor miehei* (RML) was first described in the 1970s and, in the following years, was reported for its application in food transformation [21]. RML is a highly versatile biocatalyst used in the production of chiral compounds in agrochemical and pharmaceutical industries, and in the resolution of racemates after synthesis [15, 17, 18]. However, the application of RML in industries is still limited due to its high price. RML is one of the most studied and best-known lipases, and is a single-chain α/β type protein comprising of 269 amino acid residues with a molecular size of ~31.6 kDa. Two forms of RML (Palatase 2000 L and Lipozyme RM IM) are currently commercially available from Novozymes, Denmark. Recently, the expression of RML at yields of about 50 U/mL was reported using the shake flask method [14]. Compared to other production yield of lipases such as *Rhizopus arrhizus* prolipase (140 U/mL) and *Rhizopus oryzae* lipase (500 U/mL) [9, 13], there is still a large potential to improve the yield of RML. Furthermore, the cost of this enzyme itself accounts for the majority (more than 90 %) of the total costs of the enzymatic process [2]. Thus, increasing the expression of this enzyme and reducing the production costs remain important goals in this field of research.

D. He and W. Luo contributed equally to this work.

✉ Pengmei Lv
lvpm@ms.giec.ac.cn

Dong He
hedong@ms.giec.ac.cn

Wen Luo
luowen@ms.giec.ac.cn

Zhiyuan Wang
yuanzh@ms.giec.ac.cn

Zhenhong Yuan
yuanzh@ms.giec.ac.cn

[1] Key Laboratory of Renewable Energy, Guangzhou Institute of Energy Conversion, Chinese Academy of Sciences, No. 2, Nengyuan Road, Tianhe Area, Guangzhou 510640, People's Republic of China

[2] University of Chinese Academy of Sciences, Beijing 100049, People's Republic of China

One of the most effective ways, among the various employed approaches, to achieve increased protein production is the heterologous expression of the protein, driven by a strong promoter [12, 24, 27]. The methylotrophic yeast *Pichia pastoris (P. pastoris)*, which has been proven to be an effective host for the production of both secreted and intracellular heterologous proteins, has several advantages over recombinant *E. coli* systems [3, 11]. Here, a high level of transcription of the exogenous gene can be driven by a strong, tightly regulated, and methanol-induced alcohol oxidase promoter (P_{AOX1}) [6]. However, high concentrations of methanol are toxic to *P. pastoris*, and P_{AOX1} is tightly repressed if yeast is grown on the most common carbon sources such as glucose, glycerol, or ethanol [16]. The glyceraldehyde-3-phosphate dehydrogenase promoter (P_{GAP}), a constitutive promoter, is capable of providing a comparable expression level as P_{AOX1} Similar to other constitutive promoters, P_{GAP} can ease process handling and omit the use of potentially hazardous inducers as well as provide a continuous transcription of the gene of interest [8, 28]. The combined use of two promoters in *P. pastoris* has been reported for the expression of several proteins and has been applied for enhancing the expression of one protein, and the simultaneous or sequential expression of two different heterologous proteins [4, 5, 25, 26]. However, the reasons underlying the enhancement of protein expression by the combined use of two promoters is still unclear. Furthermore, every gene promoter has specific requirements concerning expression conditions, especially related to the carbon source. Thus, balancing the requirement of the two promoters and optimizing the culture conditions is necessary to realize the optimum potential of the two promoters.

In this study, constitutive-inducible *P. pastoris* strains, which could simultaneously produce recombinant RML constitutively and inducibly, were generated by transforming the pGAPZαA-*rml* plasmid into the pPIC9 K-*rml*/GS115 strain. Four transformants among the constitutive-inducible strains that could effectively express RML driven by P_{AOX1} and P_{GAP} were screened and identified. Culture conditions for the production of RML were optimized by response surface methodology at the shake flask level. Plackett–Burman design was used to screen important factors that exert a significant effect on lipolytic activity of RML and to evaluate their extent. These factors were further optimized using the Box–Behnken design for increasing the expression of RML.

Materials and methods

Materials

Restriction enzymes and DNA polymerase were purchased from Takara Shuzo Co. Ltd. (Kyoto, Japan). *Escherichia coli* DH5α cells, *P. pastoris* GS115, and pPIC9 K and pGAPZαA plasmids were purchased from Invitrogen (Carlsbad, CA, USA). Olive oil, T4 DNA ligase, and primers for RML cloning, polymerase chain reaction (PCR) identification and quantitative PCR (qPCR) (shown in Table 1) were ordered from Sangon Biotechnology (Shanghai, China).

Plasmid construction and transformation

The *R. miehei* lipase sequence (GenBank accession No. A02536) was optimized according to the codon bias of *P. pastoris*. The optimized *rml* sequence was synthesized by Sangon Biotechnology (Shanghai, China) and cloned into pUC57 vector. A construction fragment containing the RML open reading frame, *Eco*RI at the 5′ site, and *Not*I at the 3′ site was generated by PCR using primers RML-F and RML-R (shown in Table 1) and using the plasmid pUC57-*rml* as template. PCR products were inserted into pPIC9 K

Table 1 Primers for clone, PCR identification and qPCR

Primer	Sequence (5′–3′)	Amplican size (bp)
RML-F	CCGGAATTC**GCCACC**ATGGTCCCTATTAAGCGTCAAA	1037
RML-R	ATAAGAATGCGGCCGCTTACGTGCACAACCCGGTATTTA	
AOX1-F	GACTGGTTCCAATTGACAAGC	1233
AOX1-R	TACTATTGCCAGCATTGCTGC	
GAP-F	GTCCCTATTTCAATCAATTGAA	1577
GAP-R	GCAAATGGCATTCTGACATCC	
Actin-F	TGTCCTCGGTTGAGTTTTCG	255
Actin-R	TCTAATCCCTTCCCAACGGT	
His-F	GGAAGTGGAAGGCAGAAT	86
His-R	GCTCAACTAACGGAGAC	
Zeocin-F	AAGTTGACCAGTGCCGTTCC	143
Zeocin-R	GATGAACAGGGTCACGTCGT	

Restriction endonuclease sites are underlined; Kozark sequence is in bold

and pGAPZαA vectors after double digestion with *EcoRI* and *NotI* and ligation using T4 ligase, respectively. Subsequently, the plasmids pPIC9 K-*rml* containing P_{AOX1} and pGAPZαA-*rml* containing P_{GAP} were obtained. Both plasmids contained the α factor secretion signal of *S. cerevisiae* downstream of the promoters.

pPIC9 K-*rml*/GS115 and pGAPZαA-*rml*/GS115 were transformed into *P. pastoris* GS115 competent cells according to the user manual provided by the supplier (Invitrogen, Carlsbad, CA, USA). Constitutive-inducible strains harboring P_{AOX1} and P_{GAP} were obtained by transforming the linearized vector pGAPZαA-*rml* into pPIC9 K-*rml*/GS115 competent cells and subsequent screening on YPDS plates containing 100 μg/mL zeocin. To select multicopy clones, putative clones of pGAPZαA-*rml*/GS115 and the constitutive-inducible combined strains were selected on YPD plates containing 500, 1000, 1500, and 2000 μg/mL of zeocin, whereas clones of pPIC9 K-*rml*/GS115 were selected on MD plates containing 500, 1000, and 2000 μg/mL of G418. Genomic DNA were extracted from those clones that survived the highest antibiotic concentrations and PCR was performed using *AOX1* and *GAP* primers for confirming the integration of *rml* into the genome of *P. pastoris* GS115.

Expression in *Pichia pastoris*

Each pPIC9 K-*rml*/GS115 transformant was inoculated in 100 mL BMGY and was cultured at 30 °C on a rotary shaker at 250 rpm. Cells were harvested by centrifugation at 3000×*g* for 10 min when the optical density at 600 nm (OD_{600}) reached 5.0, and pelleted cells were resuspended in 20 mL BMMY medium. The culture was grown further at 30 °C for 120 h, and methanol was added every 24 h at a final concentration of 0.5 % as the carbon source. A single pGAPZαA-*rml*/GS115 colony and the constitutive-inducible combined strains were inoculated in 10 mL YPD and grown overnight at 30 °C on a rotary shaker at 250 rpm, following which 0.1 mL of the overnight culture was inoculated in 20 mL YPD. All strains were incubated at 30 °C on a rotary shaker at 250 rpm for 120 h. In case of the constitutive-inducible combined strains, methanol was added every 24 h at a final concentration of 1 % in the combined expression phase (48−120 h) after glucose present in the culture medium was exhausted. Upon expression, hydrolytic activity of the free lipase was determined by hydrolysis of olive oil [23]. One unit (U) of lipase activity was defined as the amount of lipase that liberated 1 μmol fatty acids per minute. Transformants with highest RML lipolytic activity were further analyzed by SDS-PAGE performed on a 12 % separation gel, as described by Laemmli [10].

Absolute quantification of target gene copies was performed by qPCR conducted using a StepOnePlus™ Real-Time PCR System using ABI SybrGreen PCR Master Mix (Applied Biosystems, USA). Specific primers yielding products between 86 and 255 bp were purified by HPLC (Table 1). Genomic DNA of the strains was used as template. The copy numbers of gene *his* and *zeocin* were measured to estimate the copy numbers of P_{AOX1} and P_{GAP}, since single copy number *rml* was integrated into the genomic DNA of *P. pastoris* accompanied by single copy number *his* or *zeocin*. *Actin* (NCBI ID: 8197153) was used as the reference gene. The plasmids pPUC57-*actin*, pPIC9 K-*rml*, and pGAPαA-*rml* were constructed and used to establish the standard curves. The target gene copy numbers were calculated by absolute quantification as described by Abad et al., using the following equations [1]:

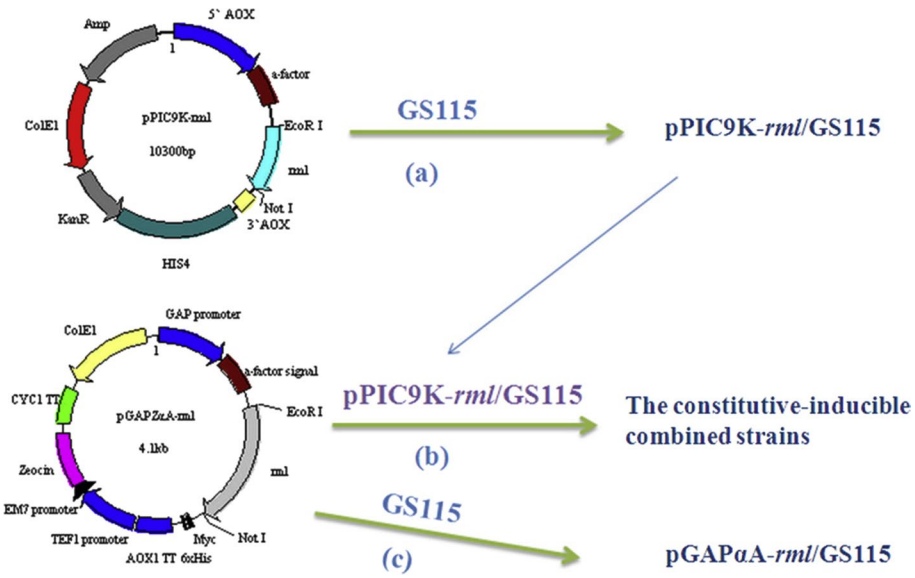

Fig. 1 Vectors construction and transformation for RML expression

$$\text{copy number}_{AOX1} = \frac{his\ (\text{copy quantity})}{actin\ (\text{copy number})}$$

$$\text{copy number}_{GAP} = \frac{Zeocin\ (\text{copy quantity})}{actin\ (\text{copy number})}$$

Response surface methodology

Response surface methodology was performed as described by Yu et al., to further increase the expression level of RML [27]. Night factors including liquid volume in flask (mL/L), casamino acids (g/L), glycerol (mL/L), oleic acid (mL/L), PTM1 (mL/L), methanol (%), Tween-80 (mL/L), YNB (g/L), and inoculation (%), which were predicted to exert an impact on the production of RML, were optimized by Plackett–Burman design to evaluate the main affecting factors. Three significant factors, screened with first-order experiments, were optimized using the Box–Behnken design for further improving the activity of RML. Three experiments were performed to compare the actual RML lipolytic activity with its predicted value obtained by the model equation to verify the genuineness of the optimum conditions obtained after response surface methodology.

Results

Generation of constitutive, inducible, and constitutive-inducible combined *P. pastoris* clones

The process of generation of the constitutive, inducible, and constitutive-inducible combined *P. pastoris* clones is shown in Fig. 1. The DNA of pPIC9 K-*rml*/GS115, pGAPZαA-*rml*/GS115, and that of the constitutive-inducible combined *P. pastoris* strains was extracted for PCR detection to confirm correct insertion of all expression cassettes. As seen in Fig. 2, one prominent band of approximately 1300 bp (lane 1) was obtained using the *AOX1* primers, whereas no band was detected using the *GAP* primers (lane 4) in strain pPIC9 K-*rml*/GS115. Further, one band corresponding to approximately 1600 bp (lane 2) was obtained using the *GAP* primers, and no band was obtained with the AOX primers in the strain pGAPZαA-*rml*/GS115. Two bands (lane 5, 6) were obtained in the constitutive-inducible combined *P. pastoris* strains using both the *AOX1* primers or the *GAP* primers. These observations were in agreement with the expected results and indicated the successful generation of constitutive, inducible, and constitutive-inducible combined *P. pastoris* clones.

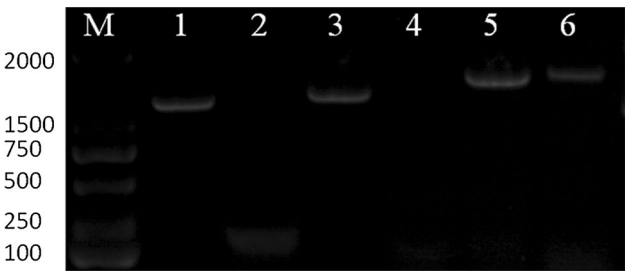

Fig. 2 PCR detection for the DNA of strains, *lane 1–3* DNA of pGAPZαA-*rml*/GS115, pPIC9 K-*rml*/GS115 and strain 3-17 as the templates, AOX primers; *lane 4–6* DNA of pPIC9 K-rml/GS115, pGAPZαA-*rml*/GS115 and strain 3-17 as the templates, GAP primers

Expression of RML in the constitutive, inducible, and constitutive-inducible strains

The expression of foreign genes in *P. pastoris* relies on different carbon source requirements between P_{AOX1} and P_{GAP}. P_{AOX1} is induced exclusively by methanol as the carbon source, whereas the expression of P_{GAP} can be induced by

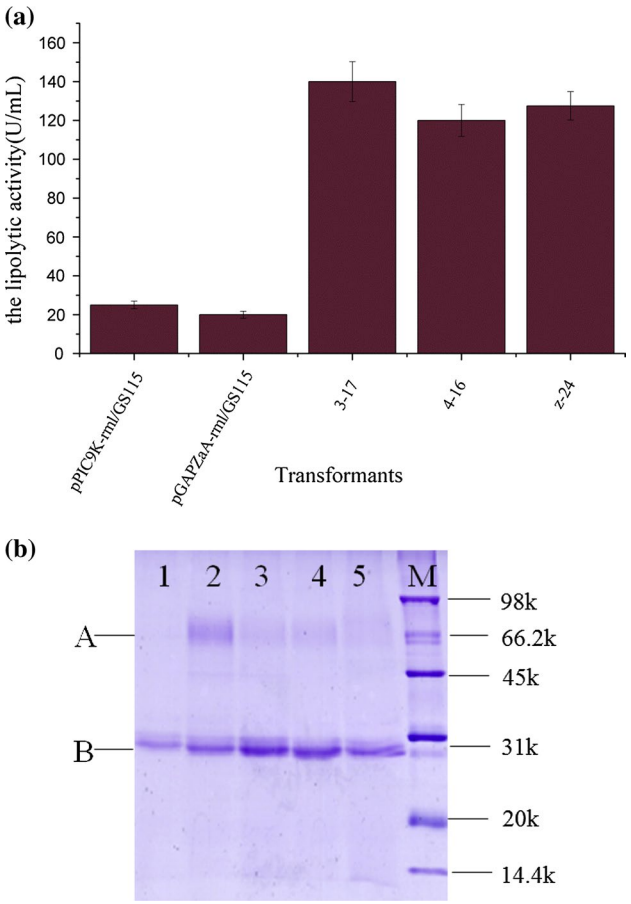

Fig. 3 The protein expression properties of the recombinant strains. **a** The lipolytic activity of transformants; **b** SDS–PAGE analysis of the secreted protein: *lane 1* pPIC9 K-*rml*/GS115; *lane 2* pGAPZαA-*rml*/GS115; *lane 3–5* three constitutive-inducible strains (3-17, 4-16, z-24). *A* glycosylated RML on its propeptide; *B* mature region of RML

various chemicals such as glucose, glycerol, oleic acid, and methanol as suitable carbon sources. Thus, a two-step carbon source addition was employed to balance the requirement of carbon sources for the combined promoter expression system. For this purpose, glucose or glycerol was used during the growth phase and the constitutive expression phase. Methanol was added as the sole carbon source in the combined expression phase after glucose or glycerol was completely exhausted. As seen in Fig. 3a, enzymatic activities of the recombinant RML constitutively expressed by the strain pGAPZαA-*rml*/GS115 and inducibly expressed by the strain pPIC9 K-*rml*/GS115 were 20 and 25 U/mL, respectively. Significantly higher enzyme activities were observed in the constitutive-inducible strains. Activity of the strain 3-17 reached about 140 U/mL, which was approximately sixfold compared to strain pGAPZαA-*rml*/GS115 or pPIC9 K-*rml*/GS115. The recombinant RML showed two bands on SDS-PAGE, as seen in Fig. 3b. One band corresponded to the mature region of RML (~30 kDa) and the second corresponded to the glycosylated RML on its propeptide (~66.2 kDa). The observed RML concentration in these bands corresponded to their enzyme activity trend.

Since P_{AOX1} cannot be induced by glucose or glycerol, almost all secreted enzyme was considered to be driven by P_{GAP} under the conditions where methanol was absent. Experiments were performed with methanol and without addition of methanol in the expression phase, to estimate the degree of increase in expression contributed by each promoter. As seen in Fig. 4, the expression of P_{AOX1} and P_{GAP} is higher in the constitutive-inducible strains 3-17, 4-16, and z-24 compared to the strains pPIC9 K-*rml*/GS115 or pGAPZαA-*rml*/GS115.

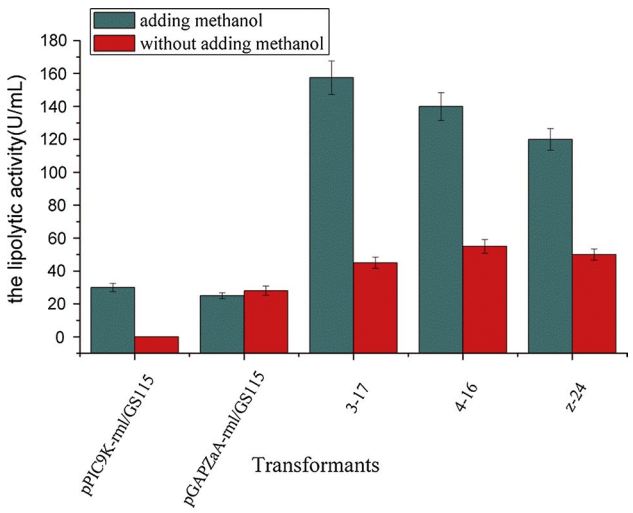

Fig. 4 The lipolytic activity of transformants under the condition with methanol and without methanol. *Bar in gray* adding methanol at the concentration of 1 %; *Bar in red* without methanol (color figure online)

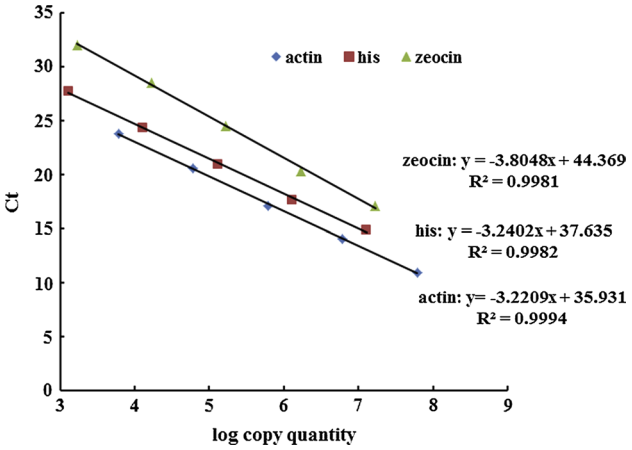

Fig. 5 Standard curves of *his*, *zeocin* and *actin* detected by qPCR. *Actin* (*inverted square in blue*), *his* (*square in red*), *zeocin* (*triangle in green*) (color figure online)

qPCR was performed to precisely determine the copy number of genes inserted in the genomes of various strains. Linear standard curves of *actin*, *his,* and *zeocin* were observed in the tested range (shown in Fig. 5), and the amplification efficiencies were 105.173, 103.532, and 94.391 %, respectively. A single peak was observed in all melting curves, indicating that the PCR products were singlets (data not shown). As shown in Table 2, the constitutive-inducible strains 3-17 and 4-16 bore identical copies of P_{AOX1} (*his*) as the parent strain pPIC9 K-*rml*/GS115, while z-24 had 4 copies of P_{AOX1}, P_{GAP} (*zeocin*) in pGAPZαA-*rml*/GS115 and the constitutive-inducible strains (3-17, 4-16, z-24) had 2, 9, 4, and 4 copies, respectively.

Optimization of culture conditions

Among the nine factors optimized by Plackett–Burman design, YNB, casamino acids, glycerol, and Tween-80 were considered to display significant effects on the activity of RML, while the other factors, whose confidence level was below 95 %, were considered insignificant. By applying regression analysis on the experimental data, a corresponding first-order model equation fitted to the data obtained from the Plackett–Burman design was as follows:

$$\text{Enzyme} = 39.58 - 4.58X_1 + 10X_2 - 9.17X_3 + 2.92X_4 - 3.75X_5 - 1.67X_6 - 8.33X_7 + 12.92X_8 - 9.25X_9$$

The three significant factors screened by Plackett–Burman design were optimized further by response surface methodology with a Box–Behnken design, and their effects on the lipolytic activity of RML were predicted by the following second-order polynomial equation:

Table 2 Copy numbers of the P_{AOX1} and P_{GAP} detected by real-time PCR

Strain	$Ct_{(his)}$	$Ct_{(zeocin)}$	$Ct_{(actin)}$	$Copy_{(AOX1)}$	$Copy_{(GAP)}$	$Copy_{(SUM)}$
pPIC9 K-*rml*	20.23 ± 0.12	–	18.14 ± 0.03	3	–	3
pGAPaA-*rml*	–	24.9 ± 0.10	20.14 ± 0.05	–	2	2
3-17	19.95 ± 0.02	21.46 ± 0.02	17.55 ± 0.10	3	9	12
4-16	17.76 ± 0.06	20.84 ± 0.04	17.91 ± 0.06	3	4	7
z-24	18.66 ± 0.04	21.77 ± 0.03	18.84 ± 0.08	4	4	8

$$\text{Enzyme activity} = 163 + 10.63A - 24.06B + 7.81AB + 18.13AC - 15.00BC - 42.75A_2 - 18.38B_2 - 37.12C_2$$

The optimal values of tested factors predicted by the model were as follows: casamino acids 16.51 g/L, glycerol 5.3 mL/L, and YNB 20.3 g/L. Final optimized medium components were: liquid volume in flask 50/250 mL, casamino acids 16.51 g/L, glycerol 5.3 mL/L, oleic acid 0.08 mL/L, PTM1 0.05 mL/L, methanol 1 %, Tween-80 0.5 mL/L, YNB 20.3 g/L, and inoculation 1 %. The maximal RML activity predicted by this model was 171.5 U/mL, and three experiments were performed to verify the accuracy of prediction under the optimal conditions. Under optimal conditions, up to 175 U/mL lipolytic activity of RML was observed in a shake flask culture, which was in excellent agreement with the predicted value.

Discussion

To increase the expression of RML, a series of measures such as codon optimization, cloning the sequence of *rml* with a propeptide, the combined use of *GAP* and *AOX1* promoters, and optimization of the culture conditions were utilized in this work.

RML could be successfully expressed in *P. pastoris* after codon optimization of *rml* with a propeptide. Codon optimization has been considered to be an effective strategy for improving the level of expression of heterologous genes that contain codons rarely used in the host organism [22]. The expression of a native gene in a heterologous host may fail due to the presence of rare codons in the gene sequence. A propeptide is decisive for RML expression, since it can lower the activation energy barrier of protein folding, which plays a crucial role in protein expression.

As seen in Fig. 3a, the combined use of the *AOX1* and *GAP* promoters significantly increased the expression of RML. This can be attributed to a positive effect on the expression level by increasing the copy number of the expression cassette. The transcription levels of the intracellular protein and secreted protein have been shown to increase greatly upon integration of multiple copies of the expression vector to an appropriate extent in *P. pastoris* [19, 20]. In this study, all constitutive-inducible combined *P. pastoris* strains were sequentially screened in selective plates containing high concentrations of G418 and zeocin to select multicopy clones. As seen in Table 2, the whole copy number of promoters including P_{AOX1} and P_{GAP} in strains pPIC9 K-*rml*/GS115, pGAPZαA-*rml*/GS115, 3-17, 4-16, and z-24 were 3, 2, 12, 7, and 8, respectively, which was in agreement with their enzyme activity trends. The copy number effect was different between the constructs that utilized the *AOX1* promoter and the *GAP* promoter [7]. Thus, to obtain the optimum copy number of the two promoters in the constitutive-inducible combined *P. pastoris* strains, a two-step screening for multicopy selection of the two promoters was necessary and proved to be effective. Another reason for the significant increase in the expression of RML by the combined use of the *AOX1* and *GAP* promoters could be that a positive correlation between gene dosage and the target protein yield may disappear after the copy number exceeds a certain value. As reported by Hohenblum et al. [7], a sharp optimum was observed when two copies were present in the construct utilizing the *AOX1* promoter. Higher enzyme activities have also been reported in the strains containing two copies of *rml* driven by P_{AOX1} compared to the strains containing one, four, or eight copies [9]. Thus, another hypothetical explanation for the enhancement of RML observed in this study could be a synergistic effect between the two promoters. As shown in Table 2, all three constitutive-inducible strains had similar copies of *AOX1* promoters as the parent strain pPIC9 K-*rml*/GS115, while expression of the protein contributed by the *AOX1* promoter in the three constitutive-inducible strains was higher than that observed in pPIC9 K-*rml*/GS115, which indicates that insertion of constructs utilizing the *GAP* promoter lead to an increase in the expression level of RML contributed by the *AOX1* promoter. However, the mechanism underlying the synergistic effect between the two promoters is still unclear. Additionally, the optimum copy number of *AOX1* promoter and *GAP* promoter, as well as their optimum ratio also remains unexplored. This result opens new perspectives for further investigations concerning the relation between copy number and synergistic effect arising due to the combined use of two promoters on protein expression in *P. pastoris*.

Expression of RML was further enhanced by optimization of conditions using the Plackett–Burman statistical design, the Box–Behnken design, and response surface methodology. YNB, casamino acids, and glycerol were shown to be significant factors and were used further to identify the absolute optimal values of each factor. In this study, casamino acids were found to be positive and crucial parameters for the expression of RML. Owing to high concentrations of amino acids and peptides in casamino acids, they are able to supply sufficient substrate material for endoproteinases and can thereby effectively avoid the degradation of heterologous proteins mediated by endoproteinases and increase protein expression. Wu et al. reported that the addition of casamino acids provides additional growth nutrients and allows cells to grow to higher concentrations, which helps increase the concentration of the secreted protein [26]. Glycerol is another crucial parameter, which is the only available carbon source during the growth phase and during constitutive expression of the protein before methanol is added. In this study, glycerol was found to exert a significant negative effect on the expression of RML, most probably by strongly repressing the activity of *AOX1* promoter. The optimum additive amount of glycerol was found to be only 0.53 %, which was slightly lower than the value in Minimal Glycerol Medium (1 %, mL/L). YNB is the basic nitrogen source for *P. pastoris* fermentation. The optimum additive amount of YNB was 20.3 g/L, which was 1.5-fold of the recommended amount mentioned in the Pichia Fermentation Process Guidelines (Invitrogen Life Technologies). This amount ensures an abundant supply of nitrogen for the cell growth and expression.

In conclusion, the current study successfully demonstrates that recombinant RML can be constitutively and inducibly expressed by the constitutive-inducible combined *P. pastoris* strains, created by transforming the expression vector pGAPZαA-*rml* into competent pPIC9 K-*rml*/GS115 cells. Following codon optimization, three transformants of the constitutive-inducible combined *P. pastoris* strains that could effectively express RML were identified. The expression level of strain 3-17 reached 140 U/mL, which was almost sixfold of that using either strain pPIC9 K-*rml*/GS115 or strain pGAPZαA-*rml*/GS115 alone. Synergistic effects of the two promoters and an increase in copy number were supposedly responsible for the enhancement of protein expression. Lipase production was successfully optimized by utilizing RSM, which led to a 1.3-fold improvement in RML lipolytic activity. In summary, the use of combined promoters in addition to several rounds of process optimization proved highly efficient for extracellular lipase production. This strategy offers a feasible approach for lowering the costs and enhancing the production of heterologous proteins in industrial applications.

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