Regulation of the Recombinant Expression of a *Rhizomucor miehei* Lipase Gene in *Aspergillus oryzae* by Hydrogen Peroxide

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Abstract: *Rhizomucor miehei* lipase (RML) is an important microbial lipase with a wide range of industrial applications. The effect of hydrogen peroxide (H_2O_2) on RML expression in *A. oryzae* transformant ONL1 was explored in this study. The results of quantitative fluorescence polymerase chain reaction (QF-PCR) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) indicated that the RML activity of the cultivated *A. oryzae* ONL1 transformants was increased by five-fold after treatment with 10 mM H_2O_2 for two hours. The H_2O_2 treatment did not appear to affect the translation of *RML* mRNA, with the elevated expression of RML being attributed to the H_2O_2 regulation of RML transcription. Therefore, hydrogen peroxide might regulate *melO* promoter-controlled heterologous gene expression at the transcriptional level. Because of the highly volatile nature of H_2O_2, a prolonged cultivation time led to a lower effect on RML activity. High RML activity within a short cultivation time could be achieved by the continuous addition of H_2O_2 in the to maintain the concentration of 10 mM in culture medium. The results of the RML activity assay and QF-PCR analysis highlighted the following strategies for the regulation of *melO* promoter-controlled RML expression in *A. oryzae*: codon optimization of RML gene sequence, signal peptide optimization, and repeated batch culture.

Key words: *Aspergillus oryzae*; *Rhizomucor miehei* lipase; hydrogen peroxide ; tyrosinase-encoding gene promoter

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双氧水对米赫根毛霉脂肪酶在米曲霉中重组表达的调控研究

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摘要： 米赫根毛霉脂肪酶 RML 是具有广泛应用价值的重要微生物脂肪酶。本研究对双氧水调控米曲霉 RML 转化子 ONL1 表达脂肪酶进行了研究。荧光定量 PCR 和 SDS-PAGE 表明，在转化子培养过程中，维持培养体系中 10 mmol/L 双氧水 2 h，使转化子 ONL1 的脂肪酶活力提高 5 倍。在双氧水处理过程中，RML 的翻译未受影响，其表达水平的提高源于双氧水对其转录水平的调控。因此，双氧水调控 melO 启动子控制的外源基因的表达体现在转录水平。由于双氧水易挥发，导致其效果低于延长培养时间。为了在较短培养时间内获得高酶活，应在培养液中持续添加双氧水（10 mmol/L）。基于米曲霉转化子脂肪酶活力的分析和 qPCR 检测，确定以下策略可用于提高米曲霉中 RML 的表达水平：脂肪酶 RML 基因的密码子优化、信号肽序列优化、连续分批培养。

关键词： 米曲霉；米赫根毛霉脂肪酶 RML；双氧水；酪氨酸酶启动子

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are a group of enzymes that catalyze diverse reactions, such as ester hydrolysis, transesterification, and ester synthesis, at a substrate-water interface or in a non-aqueous organic solvent [1]. They have a wide range of potential applications in the oleochemical, pharmaceutical, food, and detergent industries [2]; therefore, lipases produced by various microorganisms, such as *Penicillium* sp. [3], *Aspergillus* sp. [4] and *Rhizopus* sp. [5], are being thoroughly investigated.

The lipase derived from *Rhizomucor miehei* (RML) is one such enzyme. The basic characteristics of RML have been studied in detail [6, 7], and its structure has been well elucidated [8, 9]. Because of its various important applications, recombinant DNA technology has been utilized to develop various microbial production systems for RML, in order to facilitate its availability at a reasonable cost [6, 10]. The elucidation of the genome sequence, and subsequently, the genetic background, of

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Aspergillus oryzae [11] has facilitated the construction of an expression vector (pNMA-RML) for the production of RML (in *A. oryzae*) under the control of a tyrosinase-encoding gene (*melO*) promoter in this study.

melO promoter-controlled gene expression in *A. oyrzae* is suitable for submerged cultures. Under these conditions, the *melO* promoter is approximately four-fold stronger than many other powerful promoters, such as *amyB*, *glaA*, and modified *agdA* [12]. The most important advantage of *melO* promoter-controlled gene expression is the production of high-level and high-purity target protein in the culture broth of transformed *A. oryzae* [12]. However, a major disadvantage of the *melO* promoter-controlled gene expression system is the considerable amount of time required to achieve high target protein productivity. Ishida et al speculated that the *melO* gene has functions in the oxygen-scavenging system of *A. oryzae* [12]. In addition, previous researches have illustrated that the exposure of H_2O_2 to filamentous fungi could induce antioxidant enzymes (including superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase) activity [13, 14], suggesting that H_2O_2 exposure could induce a oxygen-scavenging reaction. Therefore, H_2O_2, a typical oxidant, could be theorized to regulate the transcriptional level of *melO* transcripts by controlling the *melO* promoter. If this regulation does exist for the *melO* promoter-controlled gene expression system, the cultivation time (for high productivity) could be shortened by adding H_2O_2 to the culture medium.

In this paper, the effect of hydrogen peroxide on the *melO* promoter-controlled RML expression was investigated at the transcriptional and protein levels in a submerged *A. oryzae* culture; in addition, the regulation strategies for *melO* promoter-controlled RML expression in *A. oryzae* were elucidated.

1 Materials and methods

1.1 Strains, plasmids, culture conditions, and transformation

A. oryzae niaD300, the mutant defective in the nitrate reductase gene (*niaD*$^-$), was used as the host for transformation. *E. coli* DH5α (Invitrogen, Carlsbad, CA, USA) was used to carry the expression vector pNMA-RML. pNMA-RML was constructed in our lab and contained the *melO* promoter-controlled RML gene (NCBI accession number: A02536), as well as the *niaD* gene as a selection marker (Fig.1). The signal sequence of the *A. oryzae* TAKA-amylase gene (NCBI accession number: X12726) was fused to the RML gene for secretive production. The *A. oryzae* genes (*melO*, *niaD*, and TAKA-amylase) sequences were obtained from the *A. oryzae* genome sequence published in the Dogan database (http://www.bio.nite.go.jp/dogan/Top). The protoplasts of *A. oryzae* niaD300 were prepared using lywallzyme (Guangdong Institute of Microbiology, Guangdong, China), obtained from the mycelium grown in DPY medium (2% dextrin, 1% peptone, 0.5% yeast extract, 0.5% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, pH 5.5). *A. oryzae* niaD300 was transformed according to the procedure detailed by Gomi *et al.*, with minor modifications [15]. The transformants were maintained in Czapek-Dox (CD) medium (0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 1% glucose, 1.5% agar, pH 5.5). The *A. oryzae* transformants were cultivated in MP medium (2% maltose, 2% peptone, 1% KH_2PO_4, 0.2% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, pH 6.0) for lipase production. *E. coli* DH5α was cultivated in LB medium. If required, 100 μg/mL ampicillin was added to the LB medium.

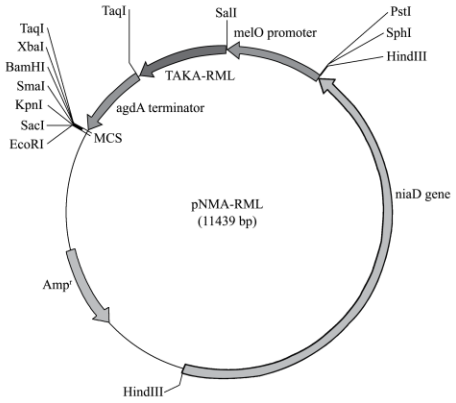

Fig.1 *RML* gene expression vector under the control of the *melO* promoter.

Note: The terminator sequence of the *A. oryzae* α-glucosidase gene (*agdA*) served as a transcription terminating signal, while *niaD* was utilized as a selection marker. The TAKA-RML lipase gene was constructed by fusing the signal sequence of TAKA-amylase to the 5′ flank of the RML gene.

1.2 Assays of transformants

Genome DNA from the *A. oryzae* transformant cultivated in CD medium was extracted and prepared

according to the method detailed by Via and Falkinham [16]. The samples were amplified by polymerase chain reaction (PCR); a PCR kit (TaKaRa Bio, Inc., Otsu, Japan) was prepared to a final volume of 25 μL, and contained 1.5 mM $MgCl_2$, 1×PCR buffer, 0.2 mM deoxy nucleotide triphosphates (dNTPs each), 0.4 μM primers (each), 1 U of Taq DNA polymerase, and 100 ng of genomic DNA. The primers used for the amplification of the 1.0 kb RML gene fragment were *rml-sense* (5′- GATCGTCGACATG ATGGTCGCGTGGTGGT-3′) and *rml-antisense* (5′- CTAGCTCGAGTTAAGTACAGAGGCCTGTGTT-3′). The reaction conditions were set as follows: denaturation at 94 ℃ for 5 min, 30 cycles of amplification at 94 ℃ for 45 s, 60 ℃ for 45 s, and 72 ℃ for 1 min, and an additional 10 min incubation at 72 ℃.

Lipase activity was measured by the alkali titration method. The substrate solution (25% olive oil, *V/V*) was emulsified in 50 mmol/L glycine-NaOH buffer (pH 8.0) with 3% PVA-124, by sonication for 5 min. The substrate emulsion was incubated at room temperature with stirring, and used within 1 h. The reaction mixture, consisting of 9.0 mL emulsion and 1.0 mL transformant culture broth, was incubated for 10 min at 40 ℃, under stirring conditions at 150 r/min (substrate emulsion was equilibrated for 5 min at 40 ℃ prior to the reaction). One milliliter of the culture broth was boiled for 10 min and added to the reaction mixture to obtain the reaction blank. The reaction was stopped by the addition of 95% ethanol (15 mL); subsequently, 15 mL of distilled water was added to the mixture. The fatty acid liberated from the substrate was titrated with 50 mmol/L NaOH, in order to standardize the pH (10.0). One lipase unit (LU) was defined as the amount of enzyme required to liberate 1 μmol fatty acid per minute from emulsified olive oil under the above-described conditions. All experiments were performed in triplicate.

1.3 Stress assays of transformants treated with hydrogen peroxide

MP medium (100 mL) was inoculated with the spores of the *A. oryzae* transformant (10^6 spores/mL) and incubated at 30 ℃ and 200 r/min in preparation for a H_2O_2 stress assay. The cells were grown for 72 h (cell dry weight = 7.5 g/L, the early exponential phase); subsequently, a stock solution of H_2O_2 was aseptically added to the culture to the desired final concentration (100 μM for initial adaptation, 10 mM for stress induction).

The culture broth was treated with 10 mM H_2O_2 for 2 h; the culture broth was then filtered through a cotton gauze for the removal of mycelia, and the filtrate centrifuged at 4000 × *g* for 5 min (4 ℃). The lipase activity of the filtrate was assayed using the alkali titration method. The filtrate was concentrated by ultrafiltration for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. SDS-PAGE was carried out in a 12% polyacrylamide gel, containing 0.1% SDS. The bands were visualized by staining with Coomassie Brilliant Blue R-250.

Real-time quantitative PCR analysis was conducted to probe *RML* mRNA abundance in the cells. Total RNA was extracted with 50 mg of transformant mycelia using RNAiso™ Plus (TaKaRa Bio, Inc.) as per the manufacturer protocols. The extracted RNA was treated with DNase I (TaKaRa Bio, Inc.) at 37℃ for 1 h. First-strand cDNA was prepared by reverse transcription of 0.5 μg total RNA, using the PrimeScript™ RT-PCR Kit (TaKaRa Bio, Inc.). The reverse transcription reaction was conducted at 37 ℃ for 15 min; the reaction was terminated by heating at 85 ℃ for 5 s. Real-time quantitative PCR assays were performed on an Applied Biosystems 7500 Real-Time PCR System, using a Fast SYBR® Green Master Mix (Applied Biosystems, Waltham, MA, USA). The *RML* mRNA level was normalized using the *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase; Dogan ID AO090011000414) reference gene. The reaction mixture (10 μL) consisted of 5.0 μL 1×Fast SYBR® Green Master Mix, 0.2 μL forward primer (10 pmol), 0.2 μL reverse primer (10 pmol), 1.0 μL cDNA, and 3.6 μL deionized water. The thermal cycling conditions were set as follows: initial denaturation at 95 ℃ for 20 s; 40 cycles at 95 ℃ for 3 s and 60 ℃ for 34 s; and the last two cycles at 95 ℃ for 15 s and 60 ℃ for 1 min, and 95 ℃ for 15 s and 60℃ for 15 s. The primers used for this reaction were rml-forward (5′-TGGATAGTCTGCCACCCCTC A-3′), rml-reverse (5′-CGAATACCACCATCAATGC TCA-3′), gapdh-forward (5′-GAAGGGGAACCCTC ATTCATC-3′), and gapdh-reverse (5′-TGGCAATGTA GGCAGTCAGG-3′). The relative quantity of the template in the samples could be determined by

comparative Ct (ABI User Bulletin). Each sample was analyzed in triplicate.

2 Results and discussion

2.1 Identification of *A. oryzae* transformants

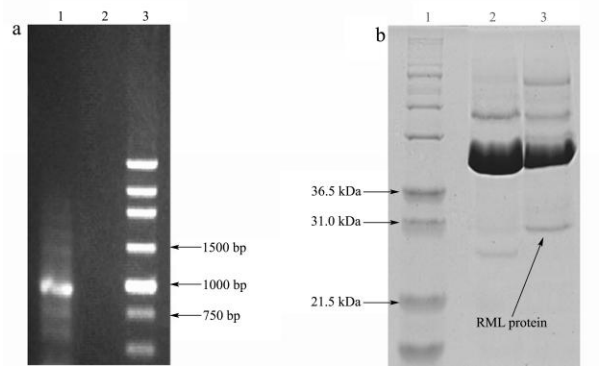

Fig. 2. PCR identification of *A. oryzae* ONL1 and SDS-PAGE analysis of the RML protein secreted by *A. oryzae* ONL1.

Note: a: PCR identification of *A. oryzae* ONL1. The rml-sense and rml-antisense primers were used for PCR analysis. Lane 1, *A. oryzae* ONL1; lane 2, *A. oryzae* niaD300 (negative control); lane 3, molecular mass marker (TaKaRa Bio, Inc.); b: SDS-PAGE analysis of the RML protein secreted by *A. oryzae* ONL1. The seventh day culture broth (25 mL) was concentrated 10-fold by ultrafiltration; filtrate (20 μL) from each sample was applied to SDS-PAGE analysis. Lane 1, molecular mass marker (Invitrogen); lane 2, *A. oryzae* niaD300 (negative control); lane 3, *A oryzae* ONL1.

Transformant candidates were cultivated in MP medium for RML production and detected based on their hydrolyzing capacity on the substrate plate. The characteristics of the *melO* promoter necessitates a long time (7 days) for the expression of RML in the transformants [12]. The 7th day culture broth of positive transformants has been observed to form clear halos on the substrate plate. The biggest halo-forming strain, designated as *A. oryzae* ONL1, was selected from among the forty halo-forming transformants, for the RML expression assay. Lipase activity was observed to reach 2.5 LU/mL in the 7th day culture broth of *A.oryzae* ONL1. The integration of the *RML* gene into the chromosome of *A.oryzae* ONL1 was identified by PCR analysis, using the genomic DNA of *A. oryzae* ONL1 as the template. A remarkable 1.0 kb band was detected in *A. oryzae* ONL1, whereas no band was detected in *A. oryzae* niaD300 (Fig. 2a), suggesting the successful integration of the *RML* gene into the genome of *A. oryzae* ONL1. The RML protein present in the *A. oryzae* ONL1 culture broth was estimated to be 32 kDa in size by SDS-PAGE (Fig. 2b), which was consistent with the results obtained by Huge-Jensen et al [6]. However, the mature RML protein was identified as a 269-amino acid polypeptide with a molecular mass of 29472 Da, which is the calculated value for the protein backbone of the mature enzyme; the difference in size may be a result of glycosylation. The SDS-PAGE results also illustrated the secretion of RML protein into the culture broth, with assistance from the signal peptide sequence of *A. oryzae* TAKA-amylase.

2.2 RML expression of *A. oryzae* ONL1 treated with H_2O_2

The *A. oryzae* RML transformant ONL1 (cultivated for 3 days) expressed RML immediately upon 2 h H_2O_2 induction (Fig.3), whereas ONL1 not subjected to induction did not express RML until 7 days post-cultivation (Fig.3); this suggested that the RML lipase could be successfully expressed in *A. oryzae*, and that H_2O_2 could effectively induce the expression of the *melO* promoter-controlled RML gene. RML activity of the *A. oryzae* ONL1 culture reached 1.0 LU/mL following treatment with 10 mM H_2O_2 for 2 h, whereas that of *A. oryzae* ONL1 culture without H_2O_2 induction was 0.2 LU/mL. Therefore, H_2O_2 addition resulted in a 5-fold increase in RML activity within 2 h. This result was consistent with the results of the SDS-PAGE analysis (Fig. 4). The band representing the RML protein (32 kDa) was darker for the sample subjected to H_2O_2 treatment, compared to that observed for the untreated sample. The results of real-time quantitative PCR analysis were also in accordance with the above conclusion. The expression level of RML-encoded transcript in the sample subjected to H_2O_2 treatment was 5.5-fold higher than that in the untreated sample (Fig. 5). The consistent fluctuation observed in the RML enzyme activity (5-fold) and *RML* mRNA (5.5-fold) values indicated that the translation process of RML was not affected by the H_2O_2 treatment. The increase in *RML* mRNA resulted in the elevation of RML enzyme activity upon being treated with H_2O_2, i.e., hydrogen peroxide regulated the transcription of the *melO* promoter-controlled RML gene. Furthermore, H_2O_2 was hypothesized to trigger increased expression of the

melO gene, based on the theory by Ishida et al that the *melO* gene functions in the oxygen-scavenging system of *A. oryzae* [12]. Therefore, the H_2O_2 regulation site in the pNMA-RML expression vector may be the *melO* promoter.

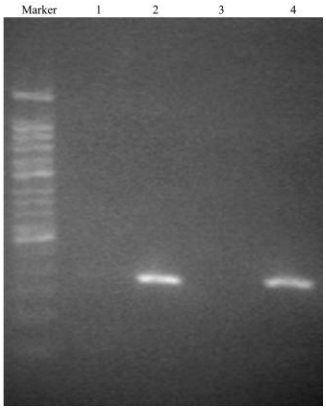

Fig.3 Initial RT-PCR identification of the effect of H_2O_2 on RML expression

Note: Marker, molecular mass marker (Invitrogen); lane 1, untreated 72 h *A. oryzae* ONL1 culture; lane 2, 72 h *A. oryzae* ONL1 culture subjected to treatment with H_2O_2 for 2 h; lane 3, untreated 120 h *A. oryzae* ONL1 culture; lane 4, *A. oryzae* ONL1 culture 168 h after H_2O_2 treatment.

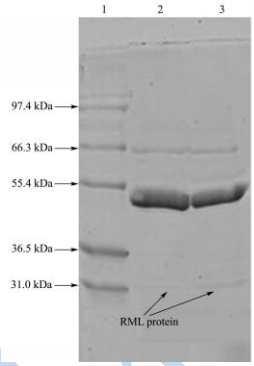

Fig.4 SDS-PAGE analysis of RML expression in *A. oryzae* ONL1 treated with H_2O_2

Note: 25 mL of the 72 h culture broth was concentrated 10-fold by ultrafiltration; 20 μL filtrate from each sample was subjected to SDS-PAGE analysis. Lane 1, molecular mass marker (Invitrogen); lane 2, untreated 72 h *A. oryzae* ONL1 culture; lane 3, *A. oryzae* ONL1 culture 72 h after treatment with H_2O_2.

On the other hand, the effect of H_2O_2 addition on *RML* mRNA expression was much weaker than when the cultivation time was prolonged. The *RML* mRNA level of the untreated *A. oryzae* ONL1 culture was 1945-fold on the 7th day, while the mRNA expression level of the 72 h *A. oryzae* ONL1 culture subjected to 2 h H_2O_2 treatment was 5.5-fold higher (Fig. 5). Li et al predicted the reason to be the complete removal of 10 mM H_2O_2 within less than 1 h after addition to the *A. oryzae* ONL1 culture [14]. Therefore, a high *RML* mRNA level could be achieved within a short cultivation time by the maintaining a H_2O_2 concentration of 10 mM, by continuously adding H_2O_2 from a stock solution.

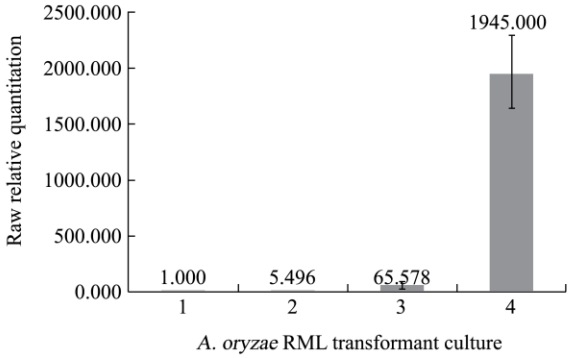

Fig.5 Real-time quantitative PCR analysis of *RML* expression in *A. oryzae* ONL1 cells subjected to H_2O_2 treatment

Note: Real-time quantitative PCR analysis. Total RNA was extracted as described in the experiment protocols and treated with DNase I. The *RML* mRNA level was normalized to that of the *GAPDH* gene. Lane 1, untreated 72 h culture; lane 2, culture obtained 72 h after H_2O_2 treatment; lane 3, untreated 5th day culture; lane 4, untreated 7th day culture. Each sample was analyzed in triplicate; the error bar represented standard deviation.

2.3 Regulation of RML expression in *A. oryzae* ONL1

The RML expression in *A. oryzae* ONL1 was characterized by another phenomenon. When the *A. oryzae* ONL1 culture was cultivated without H_2O_2 treatment, the RML activity on the 7th day was determined to be 2.5 LU/mL, which was 12.5-fold higher than that of the 72 h *A. oryzae* ONL1 culture (0.2 LU/mL). However, the *RML* mRNA level of the 7th day *A. oryzae* ONL1 culture was observed to be 1945-fold higher than that of the 72 h *A. oryzae* ONL1 culture (Fig. 5). Two possible reasons were hypothesized to account for this contradiction. One possible reason accounting for this discrepancy was that the RML protein translation was unsuccessfully conducted, despite the abundance of *RML* mRNA. This problem was explained by codon usage bias [17]. The *RML* gene might contain codons that were rarely used in the host (*A. oryzae* ONL1); therefore, the concentration of the correspondent tRNA containing anticodons complementary to those rare codons was low.

The velocity of RML protein translation reduced with the lack of tRNA assisting in the transportation of amino acids encoded by those rare codons. The constraint of codon usage bias was relieved by codon optimization of the *RML* gene sequence. Codon optimization could improve stability of the mRNA, thereby increasing the steady-state mRNA level [18]. In addition, in most cases, codon optimization could increase the level of protein expression [17]. The other possible reason for this contradiction was that the RML protein secretion might have encountered some difficulties, i.e., RML protein translation was conducted successfully, resulting in the production of a large amount of RML protein; however, the difficulty in secretion resulted in low extracellular RML activity. Many factors have been theorized to affect protein secretion, including the signal peptide. A suitable signal peptide must be selected for effective protein secretion.

This indicated that the expressional regulation of RML in *A. oryzae* ONL1 (under the control of the *melO* promoter) must be performed on the transcriptional as well as the translational/secretion level. H_2O_2 could be applied to induce a high level of *RML* mRNA within a short cultivation time at the transcriptional level, by regulating the *melO* promoter. For effective application in the *A. oryzae* ONL1 culture, H_2O_2 should be added continuously from a stock solution, and maintained at the concentration of 10 mM. A long cultivation time could also be a suitable alternative for achieving a high *RML* mRNA level in the *A. oryzae* ONL1 culture [12]. At the translational-secretion level, the *RML* sequence could be subjected to codon optimization in order to overcome codon usage bias in *A. oryzae* ONL1; this would, in turn, improve the translational level of the RML protein. Additionally, a suitable signal peptide could be selected for effective protein secretion. Alternately, the cells could be subjected to repeated batch culturing (cultivation strategy) in order to obtain a high RML protein productivity, based on the characteristics of the *melO* promoter [12].

3 Conclusion

The effect of hydrogen peroxide (H_2O_2) on *melO* promoter-controlled RML expression in the *A. oryzae* transformant ONL1 was reported in this study. The RML activity of *A. oryzae* ONL1 culture was observed to increase 5-fold (as high as the control) following treatment with 10 mM H_2O_2 for 2 h. It was also theorized that H_2O_2 might regulate the *melO* promoter-controlled heterologous gene expression at the transcriptional level. A high RML mRNA level was obtained within a short cultivation time by the continuous addition of H_2O_2, in order to maintain a concentration of 10 mM in culture medium. Based on the results of the RML activity assay and qPCR analysis, several strategies were summarized for the regulation of *melO* promoter-controlled RML expression in *A. oryzae*, including codon optimization of the RML gene sequence, selection of a suitable signal peptide, and repeated batch culture.

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