Cloning and identification of a novel tyrosinase and its overexpression in *Streptomyces kathirae* SC-1 for enhancing melanin production

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One sentence summary: Melanin production in *Streptomyces kathirae* was enhanced by introducing homogenous *melC* controlled by the *P*\(_{skmel}\) promoter.

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ABSTRACT

A 30-kDa novel tyrosinase was purified to homogeneity. The \(K_m\) for L-Dopa and L-tyrosine were determined as 0.42 and 0.25 mM. The 1231 bp (base pair) *melC* gene and its 167 bp promoter *P*\(_{skmel}\) were obtained by thermal asymmetric interlaced polymerase chain reaction based on the amino acids fragment obtained from MS results of the purified enzyme. The protein sequence of tyrosinase shows maximum identity (84%) to tyrosinase from *Streptomyces galbus*. The *melC* was introduced into *S. kathirae*. The melanin production and the transcriptional level of *melC* in recombinant *S. kathirae* [pIJ*P*\(_{skmel}\) *melC]* were about 2.1-fold and 2-fold higher than the wild-type strain, respectively. The melanin concentration was maximized at 28.8 g L\(^{-1}\).

Keywords: *Streptomyces kathirae*; melanin production; tyrosinase; enzyme properties; gene cloning; gene expression

INTRODUCTION

Melanin is a dark pigment that functions as a protective agent in diverse organisms including bacteria, fungi, plants, animals and humans (Ruan et al. 2005; Cabrera-Valladares et al. 2006; Claus et al. 2006; Wan et al. 2007; Yuan et al. 2007; Shuster and Fishman 2009). Melanin plays many self-protective roles, such as blocking UV radiation, free radical adsorption, chelating toxic iron, scavenging phenolic compounds and buffering against environmental stress. Consequently, it is widely used in medicine, pharmacology, industry and cosmetics preparation (Saxena et al. 2002; Ruan et al. 2005; Wan et al. 2007; Saini and Melo 2013). Tyrosinase (EC 1.14.18.1), a type-3 dinuclear copper-containing metalloenzyme, catalyzes the ortho-hydroxylation of monophenol to the corresponding diphenol and subsequent oxidation of the diphenol to the corresponding quinone. After a series of non-enzymatically and oxygen-involved oxidoreduction reactions, melanin is produced (Kohashi et al. 2004; Marino et al. 2011).

Currently, commercial melanin is prepared from sepia extract or by synthetic means. Recently, melanin production by microorganisms has attracted attention as an environmentally friendly and economic alternative to chemical production (Fuqua and Weiner 1993; López-Serrano, Solano and Sanchez-Amat 2004; Cabrera-Valladares et al. 2006; Selinheimo et al. 2006; Wan et al. 2007). Tyrosinase in *Streptomyces* species is encoded by...
melC2, a gene of the melanin operon (melC). Another gene of melC was found upstream of melC2 and designated melC1. Its gene product is involved in the activation and copper transmission of apotyrosinase (Chen et al. 1992) and extracellular secretion of tyrosinase in other Streptomyces (Leu et al. 1992).

Streptomyces kathirae was previously isolated by our lab and exhibits a great capacity for extracellular secretion of melanin (Guo et al. 2014). In this study, the melC operon and its promoter Pmel were cloned from S. kathirae and potential strain improvement based on the overexpression of the novel cloned homogenous melC controlled by Pmel for increasing the yield of melanin production in S. kathirae.

MATERIALS AND METHODS

Strains and plasmids

The S. kathirae strain was previously isolated and deposited at the China Center for Type Culture Collection (Wuhan, China) under accession no. M2012432. Gene cloning was conducted by pMD18-T plasmid and Escherichia coli JM109 (Takara, Dalian, China), and gene expression was performed using pJ886 plasmid and S. liiodans TK24.

Culture medium and chemicals

Escherichia coli was grown at 37°C with shaking at 160 rpm in Luria–Bertani medium (Guo et al. 2014). Streptomyces kathirae was grown at 28°C with shaking at 200 rpm in culture medium. The seed medium and optimal culture medium were prepared as previously described (Guo et al. 2014). Yeast extract-malt extract liquid medium, protoplast regeneration medium with yeast extract and agar minimal medium for sporulation were prepared as described previously (Kieser et al. 2000). Aparpycin at a concentration of 50 μg ml⁻¹ in medium was used for selection of plasmid-containing Streptomyces. The genetic manipulation reagents were purchased from Takara (Dalian, China). Other reagents were purchased from Sangon (Shanghai, China). HiPrep 16/10 Q FF Sepharose was purchased from GE Healthcare Bio-Sciences Corp. (Pittsburgh, USA).

DNA manipulation and sequencing

Plasmid and chromosomal DNA were isolated by the genomic DNA extraction kit as recommended by the manufacturer (Takara). DNA cloning and transformation of competent E. coli cells were performed as described by Sambrook, Fritsch and Maniatis (1989). Streptomyces liiodans and S. kathirae protoplast formation, transformation and regeneration were performed as previously described (Li et al. 2008). PCR was carried out as recommended by the manufacturer. DNA sequencing was performed by Sangon.

Purification of tyrosinase

For purification purposes, S. kathirae was grown in 1 L of the optimal culture medium in a 5-L fermentor (Baoxing Co., Shanghai, China) at 28°C, 1.0 rpm, pH 6.0 and 300 rpm. After 36 h of cultivation, fermentation broth was harvested by centrifugation for 30 min at 10 000 g to remove the cells and debris. All the following purification steps were carried out at 4°C. The supernatant was brought to 65% saturation concentration by addition of solid ammonium sulfate powder and allowed to stand for 12 h. The resulting precipitate was collected by centrifugation (10 000 g, 30 min), dissolved in buffer A (10 mM Tris-HCl, pH 8.0) and dialyzed against buffer A. This solution was applied to a HiPrep Q FF Sepharose column (1.6 cm × 10 cm) previously equilibrated with buffer A. The proteins were eluted by a linear gradient of NaCl (0 to 1 M) in buffer A at a flow rate of 1 ml min⁻¹. Fractions containing tyrosinase activity were pooled, dialyzed and concentrated against buffer A to 7 ml using a Stirred Ultrafiltration Cell (Millipore, USA). The purified enzyme was stored at ~20°C. The purified enzyme was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration of the purified enzyme was determined by Bradford assay, using bovine serum albumin as a standard.

Properties of purified tyrosinase

To assay tyrosinase activity, 50 μl of the enzyme solution (the final concentration of the purified enzyme was 30 μg ml⁻¹) was mixed with 5 ml of 0.05 M sodium phosphate buffer (pH 6.2) containing 10 mM L-dopa and 15 μM CuSO₄. One unit activity of the tyrosinase was defined as the amount of enzyme that catalyzes the formation of 1 μM dopachrome per minute at 30°C, where the dopachrome absorbance was measured at 475 nm (Kohashi et al. 2004).

Tyrosinase profiles assay for temperature and pH were determined at temperature ranging from 25 to 60°C at 5°C intervals at pH 7.0 and from pH 3.0 to 9.0 at 30°C in the following buffers: 0.05 M acetate buffer (pH 3.0–6.0), 0.05 M phosphate buffer (pH 6.0–8.0) and 0.05 M Tris-HCl (pH 9.0). Thermostability of tyrosinase assays was conducted at ~20°C to 60°C; pH 7.5 and pH stability of tyrosinase assays were conducted at different pH levels (ranging from 3.0 to 9.0) at 4°C. Incubation times for these assays ranged from 0 to 12 h at 3 h intervals. A control sample with no pretreatment was taken as 100%.

The effects of metal ions and chemical reagents on tyrosinase activity were determined by assaying the enzyme activity at pH 6.2, 30°C for 10 min in the presence of various reagents. The catalytic constant was determined by Lineweaver-Burk plot method using L-tyrosine and L-dopa in the range of 0.02–0.2 and 0.02–0.1 mM, respectively, at optimum pH and 30°C.

Partial amino-acid sequencing

The in-gel trypsin digestion approach was performed as previously described (Li et al. 2013). All MALDI-MS and MS/MS analyses were conducted in a mass spectrometry system (Ultraflexextreme MALDI-TOF/TOF MS, Bruker, Germany) with TOF/TOF optics. Samples were prepared by mixing 1 μl of sample with 1 μl matrix solution (0.7 mg ml⁻¹ a-cyano-4-hydroxycinnamic acid in 85% ACN containing 0.1% TFA) and spotted onto the stainless steel 192-well target plate.

Gene cloning

Based on the MS resulting protein sequence, two primers (melF, melR; see Table S1, Supporting Information) were designed and used to amplify the tyrosinase gene fragment. The 5′ and 3′ flanking regions were amplified using TAIL-PCR (Liu and Whittle 1995). Special primers and arbitrary degenerate primers are listed in Table S1 (Supporting Information). The full length gene was named melC.

Sequence analysis

The melC promoter was predicted using BDGP (http://www.fruitfly.org/seq_tools/promoter.html) (Bellen et al. 2004).
The signal peptide was predicted using Signal P 3.0 (http://www.cbs.dtu.dk/services/SignalP/) (Nielsen et al. 1997). Homology searches in GenBank were performed using the BLAST program (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/BLAST/). The molecular mass of the protein was determined using the software DNA-Man (http://www.lynnon.com/) (Lynnon, Quebec, Canada). Multiple alignments of the MelC1 and MelC2 sequence were performed with Clustal X (http://www.clustal.org/) (Larkin et al. 2007) and then manually adjusted. Phylogenetic trees were constructed using neighbor-joining algorithm in MEGA 4.0 (http://www.megasoftware.net/) (Tamura et al. 2007).

Expression of melC in S. lividans

Two putative promoters determined by BDGP were located 167 bp upstream (designated P<sub>down</sub>) and 135 bp upstream (designated P<sub>up</sub>) from the start codon of melC. The genes melC1, melC2, melC, P<sub>up</sub>melC and P<sub>down</sub>melC were individually amplified by PCR from genomic DNA of S. kathirae using primers melC1F and melC1R, melC2F and melC2R, melC1F and melC2R, P<sub>up</sub>melC and melC2RH, P<sub>down</sub>melC and melC2RH, respectively. The PCR products (melC1, melC2 and melC) were digested by HindIII and BglII and cloned into the corresponding sites of the expression vector pJRI86 to generate plasmids pJRIpermEC1, pJRIpermEC2 and pJRIpermEmelC, respectively. The PCR products P<sub>up</sub>melC and P<sub>down</sub>melC were digested by KpnI and HindIII and cloned into the corresponding sites of the expression vector pJRI86 to generate plasmids pJRP<sub>up</sub>melC and pJRI<sub>down</sub>melC (KpnI was used to remove the PermE<sup>+</sup> promoter), and then the ligation reaction was used to transform competent E. coli cells; transformants were selected in LB plates with apramycin. Plasmid DNA was extracted from several colonies, digested with corresponding restriction endonuclease and analyzed by electrophoresis in 0.8% agarose gels to verify insert size. The resultant plasmid was individually introduced into S. lividans by protoplast transformation. Transformants containing pJRIpermEC1, pJRIpermEC2, pJRIpermEmelC, pJRP<sub>up</sub>melC or pJRI<sub>down</sub>melC were selected as apramycin-resistant strains.

Introduction of melC gene into S. kathirae and confirmation of its overexpression by semi-quantitative RT-PCR

To overexpress melC gene, the pJRIpermEmelC or pJRI<sub>down</sub>melC plasmids were transformed into S. kathirae by protoplast transformation.

To confirm melC overexpression, total RNA was isolated from strains grown in optimal culture medium at 48 h. RT-PCR was conducted as described in the preceding section. To assay melC1 and melC2 expression, PCR was carried out with primers C1RT1 and C1RT2, C2RT1 and C2RT2. PCR reactions were performed using 1 μg total RNA in a 50 μl reaction mixture. After reverse transcription at 50°C for 30 min, the mixture was directly used for PCR (95°C, 2 min 1 cycle, 95°C, 30 s, 60°C, 1 min 30 cycles). Finally, 10 μl PCR products were separated on a 2.0% agarose gel with incorporated Gold view.

Batch fermentation

The batch fermentation and the melanin concentration and the reducing sugar analysis were carried out as previously described (Guo et al. 2014).

RESULTS AND DISCUSSION

Properties of the tyrosinase

The purified enzyme showed a single protein band on 12% SDS-PAGE with a molecular mass of approximately 30 kDa (Fig. S1, Supporting Information). The protein band was separated, digested and its molecular weight was determined by MS/MS. Three internal peptides, SPSFLPWHR, RFLLDFER and ASLWAPDFLGGTGR, were selected randomly, and possessed high shared amino acid identity with tyrosinase from other Streptomyces species, suggesting that the purified protein was a tyrosinase.

The maximum relative activity was observed at 45°C (Fig. 1A), which is higher than that of S. antibioticus tyrosinase (25°C) (Granata et al. 2006) and Streptomyces sp. tyrosinase (35°C) (Yoshimoto, Yamamoto and Tsuru 1985; Ito et al. 2000). Optimal tyrosinase activity was observed at pH 6.2 (Fig. 1B), which is lower than the pH 6.8–7.0 optimum reported for other tyrosinases (Lerch and Ettlinger 1972; Yoshimoto, Yamamoto and Tsuru 1985; Kohashi et al. 2004; Ito et al. 2000). Thermostability illustrated that the purified enzyme was stable below 30°C, and after incubation in 30°C for 12 h, more than 60% activity was maintained (Fig. 1C). The stability of the enzyme showed a half-life of more than 6 h at 40°C and residual activity was more than 80% after 3 h incubation at 40°C, which is much better than Streptomyces sp. KY-453 tyrosinase (Yoshimoto, Yamamoto and Tsuru 1985) that showed residual activity of 70% after 30 min incubation at 40°C. The enzyme showed good pH stability at 6.0–9.0 (Fig. 1D), with similar pH stability of Streptomyces sp. KY-453 tyrosinase (stable pH 6.0–9.5) (Yoshimoto, Yamamoto and Tsuru 1985), which was pre-incubated at the designated pH for 1 h.

Tyrosinase is a metalloenzyme, thereby the species and concentration of metal ions may play important role for the enzyme activity. Furthermore, the optimal culture medium contains high concentration yeast extract which is not economical for industrial production; thus, it is very important to find the low-cost nutrient source to substitute for yeast extract base on understanding tyrosinase properties. For the above-mentioned reason, the effects of metal ions and chemical reagents on the activity of the enzyme are presented in Table 1. The enzyme activity was inhibited by Cu<sup>2+</sup> and Ni<sup>2+</sup>. The enzyme was enhanced about 7.2-fold by Cu<sup>2+</sup> at 30 μM, but inhibition was observed when the concentration of Cu<sup>2+</sup> was above 30 μM. The enzyme activity was inhibited by Mg<sup>2+</sup> at low concentration, but slight enhancement was observed when the concentration of Mg<sup>2+</sup> was above 2 mM. Other ions enhanced the activity at low concentrations. The enzyme activity was inhibited by EDTA and SDS. The enzyme was sensitive to EDTA, while no inhibition was found in Streptomyces sp. REN-21 tyrosinase (Ito et al. 2000). Reducing agents, such as DTT, mercaptoethanol, ascobic acid, thiourea, NaNO<sub>2</sub> and L-cysteine, could strongly inhibit the enzyme activity, suggesting that reducing agents decolor the catalysis, leading to the inhibition. The activity was slightly enhanced by thiourea at low concentrations, but inhibition was observed when thiourea concentration was above 1 mM. Tween-80 and Triton X-100 could enhance the enzyme activity.

The K<sub>cat</sub>, V<sub>max</sub>, k<sub>cat</sub> and k<sub>cat</sub>/K<sub>m</sub> values of this enzyme using L-dopa as substrate were 0.42 mM, 333.28 μM mg<sup>−1</sup> min<sup>−1</sup>, 171.08 s<sup>−1</sup> and 406.37 mM<sup>−1</sup> s<sup>−1</sup>, respectively; and the values using L-tyrosine as substrate were 0.19 mM, 229.47 μM mg<sup>−1</sup> min<sup>−1</sup>, 117.80 s<sup>−1</sup> and 620 mM<sup>−1</sup> s<sup>−1</sup>, respectively. The K<sub>m</sub> value (L-dopa as a substrate) of the enzyme was 0.42 mM, which was lower than that of Streptomyces sp. REN-21 tyrosinase (4.14 mM) (Ito et al. 2000) and Streptomyces sp. KY-453 tyrosinase (5 mM)
Figure 1. Characterization of the purified tyrosinase. (A) Effects of temperature on tyrosinase activity. Symbols: filled squares, 0.05 M acetate buffer (pH 3.0–6.0); filled triangles, 0.05 M phosphate buffer (pH 6.0–8.0); filled circles, 0.05 M Tris-HCl (pH 9.0). (B) Thermostability of tyrosinase. Thermostability of the enzyme was determined by monitoring the residual activity at 45 °C in phosphate buffer (pH 6.2) after incubating the enzyme at −20 to 60 °C for 3 to 12 h in Tris-HCl buffer (pH 7.0). (C) Effects of pH on stability of tyrosinase. pH stability of the enzyme was determined by monitoring the residual activity after incubating the enzyme at 4 °C for 3 to 12 h in buffers described above at pH 3.0–9.0. The 100% relative activity was 1112.28 U mg$^{-1}$.

(Yoshimoto, Yamamoto and Tsuru 1985) and S. castaneoglobisporus tyrosinase (8.1 mM) (Kohashi et al. 2004). Notably, this value is very low, indicating a high affinity for the substrate. The substrate binding sites of tyrosinase were conserved, but residues around the substrate binding sites were different in various Streptomyces (Fig. S2, Supporting Information). Some residues may play crucial role in affecting the $K_m$ of tyrosinase; in future work, we will carry out mutagenesis study to confirm these residues.

Molecular cloning and sequence analysis

To date, many strategies have been applied to isolate flanking regions from known genomic sequences. However, these methods are time consuming and laborious. Liu and Whittier (1995) developed the TAIL-PCR strategy to successfully overcome the shortcoming of the conventional methods. Using this strategy, we successfully cloned a 1784 bp sequence from S. kathirae. A fragment of melC was amplified by PCR and comprised 104 bp (data not shown). The 5′ and 3′ flanking regions were amplified by TAIL-PCR and sequenced. Assembly of the four PCR products yielded a 553 bp 5′-non-coding region and a full-length sequence containing two open reading frames named melC1 (GenBank no. KJ868795) and melC2 (GenBank no. KJ868796), respectively (Fig. 2A). Within the 5′-non-coding region of the fragment, a possible −10 sequence (TAATTG) and the −35 sequence (TTCAAT) were determined (Fig. 2B). The gene melC1 encoded a 124-residue polypeptide and has a calculated molecular mass of 12.9 kDa. MelC1 has a ‘leader peptide’ involved in the secretion of tyrosinase and a cleavage site at the N-terminus (Ala32-Ala33) that was determined using the Signal P 3.0 program (Nielsen et al. 1997). The melC2 gene encodes 273 amino acid residues and has a calculated molecular mass of 30.8 kDa.

Phylogenetic trees were constructed based on the alignment of Streptomyces MelC1 and MelC2 (Fig. S3, Supporting Information). The results revealed that the protein sequence of MelC1 and MelC2 shows maximum identity (83 and 84%) to S. sviceus MelC1 (GenBank accession no. WP 007379786.1) and S. galbus (GenBank accession no. P55022.2), respectively.

Characterization of melC and its promoter in S. lividans

No melanin was synthesized by S. lividans [pIJPermEC1] or S. lividans [pIJPermEC2], which indicated that individually expressed MelC1 or MelC2 could not produce active tyrosinase. When MelC1 and MelC2 were coexpressed in S. lividans, S. lividans [pIJPermEmelC], S. lividans [pIJP135melC] or S. lividans [pIJPskmelC], active tyrosinase was produced because melanin was produced (Fig. 3). These results indicated that the promoter located 167 bp upstream from the start codon of melC was the true promoter of melC, because the melanin production of S. lividans [pIJPskmelC] was stronger than S. lividans [pIJP135melC]. When the melC gene transcription was controlled by 5′ upstream fragment longer than 167 bp and...
Comparison of melanin production in recombinant strains have similar promoter activities in with yeast extract which is not economical. Further study S. lividans with recombinant S. kathirae SC-1 was isolated from soil samples and optimized its culture medium by the response surface method, with a demonstrated maximized melanin concentration of 13.7 g L⁻¹ (Guo et al. 2014). In this study, the pIJPermEmelC and pIJPermEmelC plasmid was individually introduced into S. kathirae. Recombinant strains were selected as apramycin resistance, and confirmed by PCR. Strain-containing pIJ86 was used as controls. Melanin production of strains containing pIJ86 was about 13.4 g L⁻¹, almost the same as that of wild-type strains, while melanin production of S. kathirae [pIJPermEmelC] and S. kathirae [pIJPermEmelC] reached 24.9 and 28.8 g L⁻¹, about 1.9-fold and 2.1-fold higher than the wild-type strain, respectively (Fig. 4A). Almeida-Paes et al. (2012) obtained a melanin concentration of 0.07 g L⁻¹ after 9 days fermentation with Sporothrix. Lagunas-Muñoz et al. (2006) achieved a melanin concentration of 6.0 g L⁻¹ and the productivity amounted to 0.08 g L⁻¹ h⁻¹ with recombinant E. coli. Kumar et al. (2014) achieved a melanin concentration of 6.0 g L⁻¹ at 80 h with a corresponding productivity of 0.075 g L⁻¹ h⁻¹ with Pseudomonas stutzeri. In comparison with the other reported melanin-producing bacteria, strain S. kathirae [pIJPermEmelC] with a productivity of 0.21 g L⁻¹ h⁻¹ has a relatively higher melanin yield. Although the melanin production of S. kathirae [pIJPermEmelC] is high, the culture medium contains 37 g L⁻¹ yeast extract which is not economical. Further study will carried out to find low-cost nutrient source to substitute for yeast extract. The tyrosinase activity of S. kathirae [pIJPermEmelC] was about 2-fold higher than the wild-type strain during overgrowth stages (Fig. 4B). To investigate whether melanin overproduction in the recombinant strain was caused by its different growth compared with the wild-type strain, growth (Fig. 4C), reducing saccharide consumption (Fig. 4D) of the recombinant and wild-type strains were studied. Their growth was similar during all growth stages. Kinetic studies reveal that melanin production in the recombinant strain was not caused by its different growth compared with the wild-type strain. This implied that overexpression of melC gene is an efficient approach to enhance melanin production. The melanin production of S. kathirae [pIJPermEmelC] was about 1.2-fold that of S. kathirae

Expression of melC in S. kathirae

In previous studies, a high melanin-producing strain S. kathirae SC-1 was isolated from soil samples and optimized its culture medium by the response surface method, with a demonstrated maximized melanin concentration of 13.7 g L⁻¹ (Guo et al. 2014). In this study, the pIJPermEmelC and pIJPermEmelC plasmid was individually introduced into S. kathirae. Recombinant strains were selected as apramycin resistance, and confirmed by PCR. Strain-containing pIJ86 was used as controls. Melanin production of strains containing pIJ86 was about 13.4 g L⁻¹, almost the same as that of wild-type strains, while melanin production of S. kathirae [pIJPermEmelC] and S. kathirae [pIJPermEmelC] reached 24.9 and 28.8 g L⁻¹, about 1.9-fold and 2.1-fold higher than the wild-type strain, respectively (Fig. 4A). Almeida-Paes et al. (2012) obtained a melanin concentration of 0.07 g L⁻¹ after 9 days fermentation with Sporothrix. Lagunas-Muñoz et al. (2006) achieved a melanin concentration of 6.0 g L⁻¹ and the productivity amounted to 0.08 g L⁻¹ h⁻¹ with recombinant E. clo. Kumar et al. (2014) achieved a melanin concentration of 6.0 g L⁻¹ at 80 h with a corresponding productivity of 0.075 g L⁻¹ h⁻¹ with Pseudomonas stutzeri. In comparison with the other reported melanin-producing bacteria, strain S. kathirae [pIJPermEmelC] with a productivity of 0.21 g L⁻¹ h⁻¹ has a relatively higher melanin yield. Although the melanin production of S. kathirae [pIJPermEmelC] is high, the culture medium contains 37 g L⁻¹ yeast extract which is not economical. Further study will carried out to find low-cost nutrient source to substitute for yeast extract. The tyrosinase activity of S. kathirae [pIJPermEmelC] was about 2-fold higher than the wild-type strain during overall growth stages (Fig. 4B). To investigate whether melanin overproduction in the recombinant strain was caused by its different growth compared with the wild-type strain, growth (Fig. 4C), reducing saccharide consumption (Fig. 4D) of the recombinant and wild-type strains were studied. Their growth was similar during all growth stages. Kinetic studies reveal that melanin production in the recombinant strain was not caused by its different growth compared with the wild-type strain. This implied that overexpression of melC gene is an efficient approach to enhance melanin production. The melanin production of S. kathirae [pIJPermEmelC] was about 1.2-fold that of S. kathirae

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**Table 1.** Effect of metal ions and chemical reagents on the activity of the purified tyrosinase.

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</tbody>
</table>

BC represents blank control: no metal or chemical reagents.

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**Figure 2.** (A) Schematic of the melC from S. kathirae SC-1, (B) sequence of the promoter region. The putative -35 and -10 regions are indicated by italic and underlines. The consensus ribosome-binding site is indicated by underlines. The stop codon is shown by asterisk.

introduced into S. lividans, the melanin production was identical to S. lividans [pIJPermEmelC]. Furthermore, S. lividans [pIJPermEmelC] and S. lividans [pIJPermEmelC] exhibit similar melanin production in tyrosine-containing culture medium, indicating that the PermE and P_perm have similar promoter activities in S. lividans.

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**Figure 3.** Comparison of melanin production in recombinant strains S. lividans [pIJPermEC1], S. lividans [pIJPermEC2], S. lividans [pIJPermEmelC], S. lividans [pIJPerm1C] and S. lividans [pIJPermEmelC] after 48 h cultivation.
Figure 4. Changes measured parameters of melC overexpressing strain S. kathirae [pIJPermEmelC] (filled triangles), S. kathirae [pIJPerm1melC] (filled circles) and wild-type strain (filled squares) grown in optimal culture medium. (A) Melanin production; (B) tyrosinase activity; (C) mycelial dry weight; (D) reducing saccharide concentration.

[pIJPermEmelC]. One possible reason is that the homologous promoter may be more suitable than the heterologous promoter. Another possible reason is that the culture medium was optimized for S. kathirae melanin production and the medium components may be more appropriate for Pskmel promoter transcription. Elucidating the mechanism between differences in expression requires further investigation.

Transcriptional analysis of melC

RT-PCR was performed to analyze the transcriptional level of melC1 and melC2 in both wild-type and S. kathirae [pIJPermEmelC] strains. The transcriptional level of 16Sr DNA was used as control to determine the mRNA amounts of the transcriptional level of melC1 and melC2. The results showed that transcription of melC1 and melC2 in S. kathirae [pIJPermEmelC] were about 2-fold higher than that of the wild-type strain, while the transcription of the 16Sr DNA control remained almost the same level (Fig. 5).

CONCLUSIONS

In this study, the melC operon and its promoter Pskmel were cloned from S. kathirae genomic DNA. The protein sequence of tyrosinase shows maximum identity (84%) to tyrosinase from S. galbus. melC gene controlled by Pskmel was overexpressed in S. kathirae, resulting in remarkably increased melanin production. This recombinant strain produced melanin to a concentration of 28.8 g L$^{-1}$, approximately 2.1-fold higher than wild type. To our knowledge, these results set new records for melanin fermentation, and suggest that this recombinant strain is an excellent candidate for industrial-scale microbial fermentation of melanin.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSLE online.

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