Production of Melanin Bleaching Enzyme of Fungal Origin and Its Application in Cosmetics

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Abstract We screened wild fungal isolates for melanolytic activity and found that *Sporotrichum pruinosum* was the most promising of the very limited number of fungi that decolourised synthetic melanin. We used a submerged aerobic process to produce a skin depigmentation enzyme by this strain, and found that in the medium the presence of Mn^{2+} ions was necessary, the limitation of carbon source was beneficial, and Zn^{2+} ions were inhibitory. Cultivation in a stirred bioreactor required immobilization of mycelium and use of low stirring velocity. A partially purified enzyme was prepared and tested for depigmentation of human skin corneocytes and whole epidermis of phototypes III and V. This is the first study demonstrating the effective enzymatic degradation of the skin melanin rather than inhibition of its synthesis. This opens the possibility of using melanolytic enzymes in cosmetic skin lightening. © KSBB

Keywords: melanin decolouring, *melanolytic enzyme*, Sporotrichum pruinosum, *manganese peroxidase*, *skin whitening*, *depigmentation*

INTRODUCTION

Melanins are complex natural pigments, widely dispersed in animals, plants, and microorganisms. They have several biological functions including photoprotection, thermoregulation, action as free radical sinks, cation chelators, and antibiotics. Plants and insects incorporate melanins as cell wall and cuticle strengtheners, respectively [1]. The function of melanin in microbes is believed to be associated with protection against environmental stress. For example, bacteria producing melanins are more resistant to antibiotics [2], and melanins in fungi are involved in fungal pathogenesis of plants [3]. In mammals, two types of melanin can be distinguished: a dark eumelanin and a yellow to red pheomelanin [1]. Eumelanin, the more ubiquitous mammalian melanin type, is found in different regions of the human body, including the skin, hair, eye, inner ear, and brain [4].

Dermal melanins are synthesized by specialized cells called melanocytes and are located in the *stratum basale* of the epidermis. Specifically, pigments are located in organelles known as melanosomes in the form of intracellular granules, which can migrate into other epithelial cells. A crucial step in human eumelanin biogenesis is the oxidation of tyrosine by the enzyme tyrosinase to dopaquinone. In subsequent reactions, the two main building blocks of the eumelanin polymer, namely 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) are formed [1]. Although the detailed structure of human eumelanin is unknown, recent studies provide strong evidence that it is composed of aggregates of small oligomers containing about five monomer units (DHI/DHICA or their corresponding indole quinones) [4]. Eumelanins also contain about 10~15% protein [5].

Human skin is classified into six types (phototype I to VI) with respect to decreased susceptibility to sunburn, which in turn, correlates with progressively darker skin colour [6]. Skin depigmentation is applied in the medical field for disorders underlying hyperpigmentation, as well as in cosmetic skin lightening. Consequently, efforts have been made to find more effective skin-whitening agents [7]. Prevention of melanogenesis can lighten skin colour, and consequently, tyrosinase inhibitors have been extensively examined [8-16].

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Recently, it was discovered that inhibition of the transfer of melanosome into keratinocytes also results in skin lightening [17]. Such inhibitors however, may have undesirable side effects and may even be toxic [7,18-20]. For this reason, new approaches are currently being sought.

An alternative way of skin lightening is the decolouring of melanin pigment. Although melanins are very stable compounds, under special conditions chemical or photochemical degradation is possible [21] as well as biodegradation by fungi [3]. Some fungi have been reported to degrade specific melanins of different origins [22-25] and we hypothesised that fungal melanolytic enzymes could be produced for a potential application in cosmetics. Our aim was to identify the appropriate enzyme in a selected melanolytic fungus and assess its skin depigmentation ability.

MATERIALS AND METHODS

Screening of Fungi

We isolated approximately 700 fungal strains from soil and air in different geographical regions for testing of their ability to decolourise melanin. We modified Liu *et al*. [23] agar medium to 10 g glucose, 2 g malt extract, 4 g $MgSO_4 \cdot 7H_2O$, 1 g KH_2PO_4, 0.01 g $FeSO_4$, 0.005 g $ZnSO_4$, 0.2 g synthetic melanin (Sigma-Aldrich Chemie, Steinheim, Germany), and 15 g agar per litre of distilled water (pH of medium was 7.0). After autoclaving for 20 min at 121°C we poured the medium into sterile petri dishes, which were cooled, spot inoculated with fungi, and incubated at room temperature (~24°C) for several days. The extent of decolouring of the dark medium under and around the fungal colony was observed and diameter of the zone was measured daily. The active strains were identified taxonomically.

Enzyme Production in Shaken Cultures

We selected the fungus *Sporotrichum pruinosum* for this study for its potential decolouring activity. The fungus grew in shaken flasks to produce the melanolytic enzymes. We modified the culture medium from Tomaževič and Perdih [26] to 10 g glucose, 0.2 g yeast extract, 0.07 g veratryl alcohol, 3.0 g tartaric acid, 1 g Tween 80, 0.2 g KH_2PO_4, 0.146 g $CaCl_2 \cdot 2H_2O$, 0.157 g $(NH_4)_2HPO_4$, 0.05 g $MgSO_4 \cdot 7H_2O$, 42.5 mg $ZnSO_4 \cdot 7H_2O$, 3.38 mg (0.02 mM) $MnSO_4 \cdot H_2O$, 7 mg $CoCl_2 \cdot 6H_2O$, 7 mg $CuCl_2 \cdot 2H_2O$, 0.54 mg $FeCl_3$, and 0.9 mg NaCl per litre of distilled water, and adjusted the pH of the medium to 4.5 with NaOH. Next, we poured 100 mL aliquots into 500 mL flasks followed by autoclaving at 121°C for 20 min. The inoculum was prepared from fungal cultures grown on malt extract agar slants at 30°C for 10 days. The spores from one slant were suspended in 25 mL of sterile water and the obtained suspension (10^6~10^7 spores/mL) was used in aliquots of 5 mL to inoculate 100 mL of the medium. We incubated the flasks on a rotary shaker (New Brunswick Scientific, Edison, NJ, USA) at 30°C and 100 rpm for up to 10 days. Duplicate samples were collected periodically by filtering the culture broth through "black ribbon" filter paper (Schleicher & Schuell, Dassel, Germany), followed by testing the filtrate for melanolytic activity. We optimised the medium during the experiments by varying the content and concentrations of glucose, Mn^{2+}, and trace elements (Zn^{2+}, Co^{2+}, Cu^{2+}, and Fe^{2+}).

Enzyme Production in Bioreactor

To scale up the process we produced the melanolytic enzymes within an Infors ISF-100 stirred bioreactor vessel (Infors, Bottmingen, Switzerland) of 5 L working volume. When optimising the process parameters we mounted a marine type impeller to the bottom of the bioreactor vessel and an autoclavable cylindrical plastic net on the inner side to immobilize the fungal mycelium. The optimised medium was poured into the bioreactor vessel, autoclaved at 121°C for 20 min and after cooling it was inoculated by 50 mL of *S. pruinosum* spore suspension (10^6~10^7 spores/mL). We performed the fermentation at 30°C, aerated initially at 0.6 vvm followed by an increase to 1 vvm after 48 h due to a steady decrease in dissolved oxygen concentration. The agitation rate was 80 rpm throughout the process and pH and the dissolved oxygen concentration in the bioreactor were measured on-line by means of corresponding electrodes. About 20 mL of the broth were taken each day, filtered, and the filtrate analysed for soluble proteins, reducing sugars and enzyme activity. After 10~11 days, the process was stopped and the broth was filtered through a black ribbon filter paper. The filtrate was subsequently frozen at −20°C.

Preparation of Enzyme Powder

To remove extracellular polysaccharides, the filtrate was frozen overnight, thawed and after filtration (black ribbon filter paper) the precipitate was discarded. The filtrate was filtered through a 0.22 μm membrane (Millipore, Bedford, MA, USA) and then concentrated by an ultrafiltration device Viva-flow (Vivascience, Hannover, Germany) with 10 kDa cut-off membrane. The retentate was desalted by dialysis and lyophilised (Micro Modulyo, Edwards, Cambridge, UK) to produce an active enzyme powder for skin-lightening tests.

Analyses

To quantify soluble proteins we used the bicinchoninic acid (BCA) method according to the manufacturer's instructions (Sigma-Aldrich Chemie). In addition, we measured reducing sugars according to Miller [27] with some modifications. Briefly, a sample solution was mixed (1:1) with a reagent consisting of an aqueous solution of 1% 3,5-dinitrosalicylic acid, 1.6% NaOH, and 2 mM EDTA (added to prevent interference of Mn^{2+} ions from the medium). The mixture was incubated at 100°C for 5 min and diluted with distilled water (1:2.5). We measured the absorbance at 540 nm against the blank prepared in the same way but replacing the sample solution with distilled water.

We measured melanolytic activity as the activity of man-

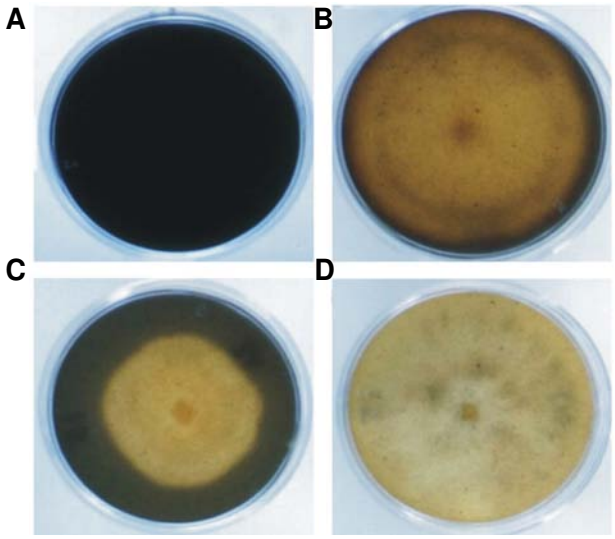

Fig. 1. Screening fungi for decolouration of synthetic melanin in agar plates: (A) control, (B) *Stropharia coronilla*, (C) *Geotric-hum* sp., (D) *Sporotrichum pruinosum*.

ganese peroxidase using 2,6-dimethoxyphenol (DMP) as a substrate [28]. The reaction was conducted in a malonate buffer at pH 4.5 with 80 mM DMP in the presence of $MnSO_4$. We added hydrogen peroxide to initiate the reaction and we measured the synthesis of the product spectrophotometrically at 468 nm continuously over the first 120 sec. The amount of the enzyme producing 1 μmol of the product per minute had one unit of enzyme activity.

To study the stability we examined the enzyme activity over seven days by incubating it in malonate buffer (pH 4.5 and 4, 20, or 30°C). Aliquots of the enzyme solution were withdrawn in time intervals (see Fig. 3) and the melanolytic activity was measured.

Skin-whitening Experiments

We first tested the whitening effect of the enzyme on corneocytes of human skin epidermis (phototype III). For this purpose the uppermost layer of the epidermis (*stratum corneum*) was removed from the skin using an adhesive band. The thin layer of corneocytes remaining on the band was incubated in 50 μg/mL of the melanolytic enzyme and phosphate-buffered saline (PBS) at pH 7.0 and 37°C for 24 h. We then rinsed the corneocytes with PBS and then stained them according to Fontana-Masson protocol [29] with colloidal silver prior to microscopic observation. We then quantified the melanin lightening in percentage using the computer image-processing package Visilog 5.4 (Noesis, Les Ulis, France). In addition, we counted the number of melanin granules per unit area using the statistics Statgraphic Plus 5.1 (Sigma-Plus, Levallois-Perret, France). The results were presented as a melanin content index, which is defined as the ratio between the average number of melanin granules in a corneocyte and the average surface area of a corneocyte.

To test the effect of the enzyme on skin we prepared an enzyme solution in PBS buffer, we sterilized the solution by filtration (0.22 μm). Pieces of human skin (phototype V - black skin) were obtained from biopsies of plastic surgery. The epidermis was separated from the dermis by incubation in 2 N NaBr for 105 min at 37°C, and placed into the survival medium to preserve optimal conditions for the biopsy (following the instructions of the research centre BIO-EC, Clamart, France). The enzyme solution was added, resulting in a final melanolytic enzyme concentration of 50 μg/mL. We then incubated the samples at 37°C for 24 h. We rinsed the pieces of epidermis with PBS at 37°C and examined them with an optical microscope for depigmentation. We compared the degree of whitening by image analysis as stated above. We used the epidermis incubated in buffer without the enzyme as a negative control, and the epidermis incubated with a known chemical whitening agent according to Kligman and Willis (containing 0.1% tretinoin, 5.0% hydroquinone, 0.1% dexamethasone, and hydrophilic ointment) [30] as a positive control.

To examine whether the melanolytic enzyme could act as inhibitor of melanin synthesis we tested its eventual effect on the dopa oxidase, the key enzyme in melanogenesis. The separated epidermis (as the source of the dopa oxidase) was fixed by formol buffer (pH 7.0, 0.05 M), rinsed and incubated for 1 h in the presence of L-dopa (3,4-dihydroxy-L-phenylalanine) and the melanolytic enzyme, 0.005% (w/w). The samples, incubated in PBS, served as a negative control, while the samples incubated in 0.015% (w/w) kojic acid (a dopa oxidase inhibitor), served as a positive control. After incubation, spots of melanin were counted by optical microscopy.

RESULTS

Screening of Fungi for Melanolytic Activity

We screened approximately 700 fungal strains (commonly present in air and soil) on agar plates for melanolytic activity and found only five of the fungi tested to have the ability to decolourise the pigment. They belong to the following four species: *S. pruinosum*, *Stropharia coronilla*, *Geotrichum* sp., and a wood fungus with a blue coloured mycelium that could not be taxonomically identified due to lack of sporulation. We report the decolouring of agar plates containing synthetic melanin by the three most active fungi in Fig. 1. We found *S. pruinosum* to completely decolourise the pigment over the whole agar plate (Fig. 1D), and in turn selected it for our study of melanolytic enzyme production in an aerobic submerged culture.

Development of Enzyme Production Process

We introduced the medium with the synthetic melanin (as used in agar plates) for the development of submerged melanolytic enzyme production, but it did not yield any promising results. Similarly, the quantification of mel-

Table 1. Concentration and purification steps and their efficiency

	Quantity (mL)	Enzyme activity (U/L)	Total activity (U)	Yield (%)	Soluble proteins (mg/L)	Specific activity (U/g)
Filtrate	4,090	104	425	100.0	160	650
Retentate	190	2,019	384	90.2	860	2,348
Lyophilysate	700[a]	487[b]	341	80.2	236[c]	2,064

[a]In mg, [b]in U/g, [c]in mg/g.

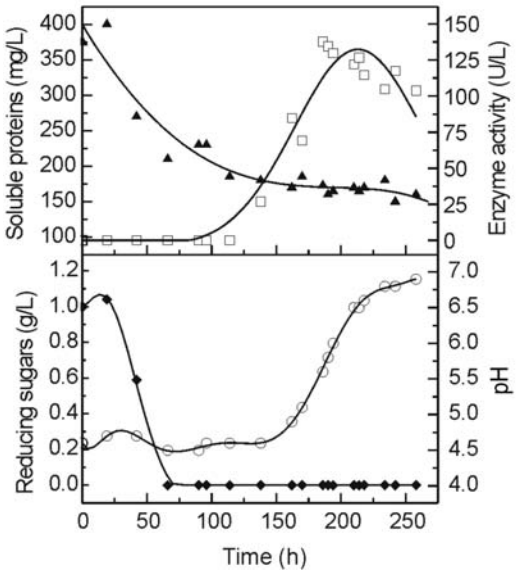

Fig. 2. Time course of fermentation parameters during enzyme production in a stirred bioreactor (□, enzyme activity; ▲, soluble proteins; ○, pH; ◆, reducing sugars).

anolytic activity by spectrophotometric analysis of melanin decolouring by the culture filtrate was unsuccessful. *S. pruinosum* is an anamorph of the fungus *Phanerochaete chrysosporium*, and according to Butler and Day [24] manganese peroxidase (one of its ligninolytic enzymes) is responsible for the degradation of fungal melanin by this fungus. Accordingly, we adopted a cultivation process, which induced the synthesis of ligninolytic enzymes [26]. The enzymatic activity was measured using DMP as a substrate [28] in place of melanin and hence, a production process was developed. The initial medium was further optimised, since we found that an increase in enzymatic activity was achieved after the glucose concentration was reduced from 10 to 1 g/L, and the Mn^{2+} concentration increased from 0.02 to 0.2 mM. Higher concentrations of Mn^{2+} ions (1 and 2 mM) and the addition of other trace elements (especially Zn^{2+} ions) diminished enzyme activity. Therefore, in the optimised medium we adopted new concentrations of glucose (1 g/L) and Mn^{2+} (0.2 mM), and we omitted Zn^{2+}, Co^{2+}, Cu^{2+}, and Fe^{3+}. All other constituents remained in the initial concentrations.

With the optimised medium, the process was scaled-up and carried out in a 5-L stirred bioreactor. We found that a simple transfer was not possible since the fungus did not form pellets in the bioreactor as it did in the shaken flasks. Furthermore, the mycelium grew on the walls and bottom of bioreactor vessel, as well as on the impeller. Under such conditions, the mycelium could not be uniformly aerated and no enzyme production could be detected. Because the fungi had a tendency to attach to solid surfaces, we inserted a cylinder made of a sterilisable plastic net mounted parallel to the walls of the vessel. The mycelium attached itself on the net and grew as a thin film. The enzymatic activity could only be detected in an appreciable amount when a gentle agitation of 80 rpm by a marine type impeller was performed.

We could not monitor the consumption of dissolved oxygen, which is an indicator of fungal growth, past the initial lag phase of about 50 to 60 h because the mycelium grew on the electrode. The time courses of the measured parameters during enzyme production in a bioreactor are depicted in Fig. 2. The concentration of reducing sugars decreased steadily to the point of non-detection at 65 h. The pH of the broth reached its minimum at the same time and subsequently began to rise. We first observed enzymatic activity at about 95 h, which was 30 h after the non-detection of sugar and minimum pH. The maximal activity of nearly 140 U/L was attained at about 200 h. Up till about 100 h we observed a steady decrease in soluble proteins which corresponded to the consumption of the proteins in the medium. Later, the production of proteins, including enzymes, overcame the decrease of the soluble protein amount.

Enzyme Preparation and Skin Depigmentation

We harvested the extracellular enzyme produced in the bioreactor at the time of maximal activity. Next, after elimination of extracellular polysaccharides we partially purified, and concentrated the enzyme by ultrafiltration, dialysis, and lyophilysation to obtain an enzyme preparation for skin-whitening tests (Table 1). In the ultrafiltration step we observed a 95% reduction in the volume, while the enzyme activity increased by nearly the same factor. Only a 10% decrease in the total activity was observed in this step. We obtained a yield of ~80% after dialysis and lyophilysation steps. We judged (by the specific activity of the soluble protein) the enzyme preparation to be more than three times purified compared to the initial culture filtrate (Table 1).

For determination of enzyme stability we incubated the enzyme preparation in a buffer solution at 4, 20, and 30°C. Over the course of seven days, we observed a steady decrease in stability at each temperature (Fig. 3). Enzyme incubated for one week at 4 and 20°C retained 92 and 81% of

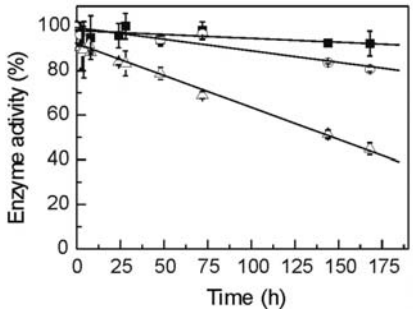

Fig. 3. Stability of melanolytic enzyme solution at different incubation temperatures (■, 4°C; ○, 20°C; △, 30°C).

the initial activity, respectively. At 30°C half-life of the enzyme activity was 6 days.

We tested the enzyme powder for skin depigmentation ability; however, the Mn^{2+} ions and H_2O_2, necessary in the enzyme activity measurements, were omitted since their use in cosmetics is unacceptable. Despite this, the experiments on corneocytes from human skin epidermis of phototypes III showed promising results. After 24 h of incubation at 37°C with the melanolytic enzyme (50 μg/mL) the melanin content in the corneocytes decreased by 50% compared to the control sample (Fig. 4).

The depigmentation activity of the produced enzyme was confirmed with the samples of skin epidermis phototype V. We observed the fungal melanolytic enzyme to decolourise the human eumelanin to the same degree as the chemical formulation of Kligman and Willis [30].

It is known that the Kligman and Willis skin whitening formulation works by inhibiting melanin synthesis. We wanted to further discover whether the fungal melanolytic enzyme also had the same activity. Therefore, the enzyme was incubated with L-dopa in the presence of the epidermis as a source of a dopa oxidase. We found no difference between the negative control (buffer) and the melanolytic enzyme, while kojic acid, a known inhibitor of dopa oxidase [7,31] inhibited L-dopa oxidation for 98%. We confirmed that the melanolytic enzyme did not inhibit melanin synthesis.

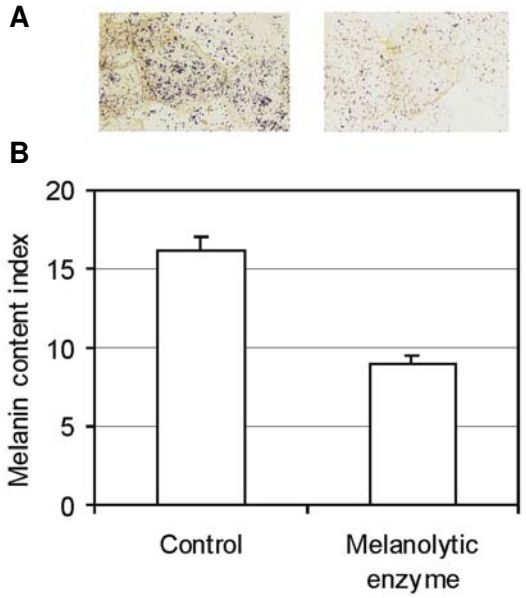

Fig. 4. Lightening of melanin in corneocytes (phototype III): (A) photo left, control; photo right, corneocytes after 24 h incubation with melanolytic enzyme at 37°C. (B) Quantification of lightening by determination of melanin content index.

DISCUSSION

In this study we investigated the possibility of using an enzyme for skin depigmentation. Previous reports have studied microbial degradation of melanins for different purposes. Luther and Lipke [22] attempted to elucidate the natural degradation of melanins from different sources by soil microorganisms and found an *Aspergillus fumigatus* strain with significant melanolytic activity. In several studies researchers attempted to degrade melanins of a microbial origin as a means of decolourising wastewater from antibiotic production [23] and as a biological control against melaninized plant pathogenic [24] or wood degrading [25] fungi. While bacteria were not able to degrade melanin, some fungi were effective in doing so [25]. From these studies, various species of fungi were reported as the species of choice including an *Acrostaphylus* sp. belonging to fungi imperfecti [23], an ascomycete *Galactomyces geotrichum* [25] and a ligninolytic white rot fungus *P. chrysosporium* [24], which suggested that an appropriate melanolytic enzyme of fungal origin may exist. We then decided to begin the study with a large scale screening experiment. However, only a very limited number of strains were able to decolourise synthetic melanin. Among these *S. pruinosum* (a wood decaying fungus, which degrades lignin and causes white rot) was the most promising. Previous studies reported the whole melanoprotein is structurally related to humic acid and lignin [5] and therefore, it is not surprising that the ligninolytic and the melanolytic activities are closely related. Our results are consistent with those of Butler and Day [24], which reported that melanin (in their study of fungal origin) was degraded by white rot fungi. The species selected in their study, *P. chrysosporium*, is actually a teleomorph of *S. pruinosum*. The same authors found that the enzyme responsible for fungal melanin degradation by *P. chrysosporium* was manganese peroxidase, which is one of the ligninolytic enzymes. Similarly, we found that our enzyme, which expressed manganese peroxidase activity, could decolourise human skin melanin (even though its composition differs from that of fungal melanins). The use of a medium to induce ligninolytic enzyme synthesis and measurement of manganese peroxidase activity as an indicator for melanolytic activity proved to be appropriate. Moreover, enzyme samples with higher activity were also more effective in decolourising

human eumelanin. While enzyme production in shaken flasks was accomplished after introduction of a suitable medium, the scaling-up into a stirred bioreactor demanded more effort. The fungus, which in nature grows on wood, tended to attach itself in clumps onto solid surfaces. To assure homogeneous oxygenation and enzyme activity, mycelium immobilization in combination with gentle stirring of the medium was necessary. Under these conditions, sufficient amounts of enzyme were produced in a bioreactor and a partially purified, concentrated powder could be prepared. The enzyme was rather stable in the buffer solution even at 20°C and its stability could be further improved by the appropriate formulation of a cosmetic preparation. The enzyme was then used for skin depigmentation tests. The usual addition of Mn^{2+} ions and a small amount of H_2O_2, which were present during enzyme activity measurement on DMP, had to be omitted and the enzyme was applied alone. Contrary to expectations, the depigmentation of human skin samples with the enzyme was effective without applying any of the two additives. It is possible that these substances are already present in the skin or that some other ion and oxidant fulfils their role.

CONCLUSION

Here, we present the first report of a fungal enzyme used for bleaching of human eumelanin in skin. The screening of fungi for melanolytic activity revealed an active *S. pruinosum* strain, which cultivated under properly selected culture conditions (suitable medium composition, mycelium immobilisation, gentle stirring) could produce an extracellular melanolytic enzyme in a stirred bioreactor. The concentrated and partially purified enzyme could successfully depigment human skin samples. Our results demonstrated that contrary to the known whitening agents, the enzyme does not act as an inhibitor of pigmentation but rather as a real whitening agent of the already formed melanin. This new approach of using a melanin decolouration enzyme appears to be promising and is important because the enzyme is of biological origin, and will be more acceptable for cosmetic purposes than chemical formulations. Thus, it opens a new way of using the melanolytic enzymes in skin whitening.

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REFERENCES

1. Riley, P. A. (1997) Melanin. *Int. J. Biochem. Cell Biol.* 29: 1235-1239.
2. Lin, W. P., H. L. Lai, Y. L. Liu, Y. M. Chiung, C. Y. Shiau, J. M. Han, C. M. Yang, and Y. T. Liu (2005) Effect of melanin produced by a recombinant *Escherichia coli* on antibacterial activity of antibiotics. *J. Microbiol. Immunol. Infect.* 38: 320-326.
3. Butler, M. J. and A. W. Day (1998) Fungal melanins: a review. *Can. J. Microbiol.* 44: 1115-1136.
4. Clancy, C. M. R. and J. D. Simon (2001) Ultrastructural organization of eumelanin from *Sepia officinalis* measured by atomic force microscopy. *Biochemistry* 40: 13353-13360.
5. Neumüller, O. A. (1985) *Römpps Chemie-Lexikon*. 8th ed., Vol. 4., pp. 2528-2529. Franckh'sche Verlagshandlung, Stuttgart, Germany.
6. Young, A. R. (2006) Photobiology of melanins. pp. 342-353. In: J. J. Nordlund, R. E. Boissy, V. J. Hearing, R. A. King, W. S. Oetting, and J. P. Ortonne (eds.). *The Pigmentary System: Physiology and Pathophysiology*. Blackwell Publishing, Oxford, UK.
7. Rendon, M. I. and J. I. Gaviria (2005) Review of skin-lightening agents. *Dermatol. Surg.* 31: 886-889.
8. Terao, M., K. Tomita, T. Oki, L. Tabe, M. Gianni, and E. Garattini (1992) Inhibition of melanogenesis by BMY-28565, a novel compound depressing tyrosinase activity in B16 melanoma cells. *Biochem. Pharmacol.* 43: 183-189.
9. Cabanes, J., S. Chazarra, and F. Garcia-Carmona (1994) Kojic acid, a cosmetic skin whitening agent, is a slow-binding inhibitor of catecholase activity of tyrosinase. *J. Pharm. Pharmacol.* 46: 982-985.
10. Arai, N., K. Shiomi, S. Takamatsu, K. Komiyama, M. Shinose, Y. Takahashi, Y. Tanaka, Y. Iwai, J. R. Liu, and S. Omura (1997) Amphistin, a new melanogenesis inhibitor, produced by an actinomycete. *J. Antibiot.* 50: 808-814.
11. Dooley, T. P. (1997) Topical skin depigmentation agents: current products and discovery of novel inhibitors of melanogenesis. *J. Dermatolog. Treat.* 8: 275-279.
12. Shimizu, K., R. Kondo, K. Sakai, S. H. Lee, and H. Sato (1998) The inhibitory components from *Artocarpus incisus* on melanin biosynthesis. *Planta Med.* 64: 408-412.
13. Sugimoto, K., T. Nishimura, K. Nomura, K. Sugimoto, and T. Kuriki (2004) Inhibitory effects of alpha-arbutin on melanin synthesis in cultured human melanoma cells and a three-dimensional human skin model. *Biol. Pharm. Bull.* 27: 510-514.
14. Boissy, R. E., M. Visscher, and M. A. DeLong (2005) DeoxyArbutin: a novel reversible tyrosinase inhibitor with effective *in vivo* skin lightening potency. *Exp. Dermatol.* 14: 601-608.
15. Kim, Y. J. and H. Uyama (2005) Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition mechanism and perspective for the future. *Cell. Mol. Life Sci.* 62: 1707-1723.
16. Cho, Y. H., J. H. Kim, S. M. Park, B. C. Lee, H. B. Pyo, and H. D. Park (2006) New cosmetic agents for skin whitening from *Angelica dahurica*. *J. Cosmet. Sci.* 57:

11-21.
17. Seiberg, M., C. Paine, E. Sharlow, P. Andrade-Gordon, M. Costanzo, M. Eisinger, and S. S. Shapiro (2000) Inhibition of melanosome transfer results in skin lightening. *J. Invest. Dermatol.* 115: 162-167.
18. del Giudice, P. and P. Yves (2002) The widespread use of skin lightening creams in Senegal: a persistent public health problem in West Africa. *Int. J. Dermatol.* 41: 69-72.
19. Matsubayashi, T., T. Sakaeda, T. Kita, Y. Kurimoto, T. Nakamura, K. Nishiguchi, T. Fujita, F. Kamiyama, A. Yamamoto, and K. Okumura (2003) Intradermal concentration of hydroquinone after application of hydroquinone ointments is higher than its cytotoxic concentration. *Biol. Pharm. Bull.* 26: 1365-1367.
20. Al-Saleh, I., N. Shinwari, I. El-Doush, G. Billedo, M. Al-Amodi, and F. Khogali (2004) Comparison of mercury levels in various tissues of albino and pigmented mice treated with two different brands of mercury skin-lightening creams. *Biometals* 17: 167-175.
21. Robbins, C. R. (1994) *Chemical and Physical Behaviour of Human Hair.* 3rd ed., pp. 142-150. Springer Verlag, New York, NY, USA.
22. Luther, J. P. and H. Lipke (1980) Degradation of melanin by *Aspergillus fumigatus*. *Appl. Environ. Microbiol.* 40: 145-155.
23. Liu, Y. T., S. H. Lee, and Y. Y. Liao (1995) Isolation of a melanolytic fungus and its hydrolytic activity on melanin. *Mycologia* 87: 651-654.
24. Butler, M. J. and A. W. Day (1998) Destruction of fungal melanins by ligninases of *Phanerochaete chrysosporium* and other white rot fungi. *Int. J. Plant Sci.* 159: 989-995.
25. Rättö, M., M. Chatani, A. C. Ritschkoff, and L. Viikari (2001) Screening of micro-organisms for decolorization of melanins produced by bluestain fungi. *Appl. Microbiol. Biotechnol.* 55: 210-213.
26. Tomaževič, E. and A. Perdih (1996) Determination of intrapellet ligninolytic activity. *Folia Microbiol.* 41: 499-501.
27. Miller, G. L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31: 426-428.
28. Mohorčič, M., S. Teodorovič, V. Golob, and J. Friedrich (2006) Fungal and enzymatic decolourisation of artificial textile dye baths. *Chemosphere* 63: 1709-1717.
29. Sheehan, D. C. and B. B. Hrapchak (1980) *Theory and Practice of Histotechnology*. 2nd ed., pp. 277. YB Medical Publishers, London, UK.
30. Kligman, A. M. and I. Willis (1975) A new formula for depigmenting human skin. *Arch. Dermatol.* 111: 40-48.
31. Nohynek, G. J., D. Kirkland, D. Marzin, H. Toutain, C. Leclerc-Ribaud, and H. Jinnai (2004) An assessment of the genotoxicity and human health risk of topical use of kojic acid [5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one]. *Food Chem. Toxicol.* 42: 93-105.