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Improvement of bilirubin oxidase productivity of *Myrothecium verrucaria* and studies on the enzyme overproduced by the mutant strain in the solid-state fermentation

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Bilirubin oxidase has applications in the health and environmental sectors. Hence, several attempts have been made to increase enzyme yields. However, improvements were not very high. We report here the development of a mutant strain of *Myrothecium verrucaria* by using UV-rays, which produced 28.8 times more enzyme compared with the parent and was higher than the yields reported in earlier submerged cultures. The mutant strain produced 35.6 times more enzyme than the parent in solid-state fermentation, which is better than that previously reported for a solid-state fermentation process. The specific activity of the enzyme produced by the mutant was higher than that of the parental enzyme. Bilirubin oxidase from both strains showed an optimum activity at pH 7 and 40°C. However, the time required to inactivate half of the initial enzyme activity at 60°C was much higher in the case of the enzyme obtained from the mutant compared with the parental enzyme. The improved thermostability of the enzyme from the mutant strain could be due to the point mutations induced during the UV irradiation, since there was no change in the mass of the enzyme compared with the parental enzyme. The bilirubin oxidase of the mutant strain degraded the bilirubin faster than the enzyme obtained from the parent under similar conditions. Faster activity of the enzyme obtained from the mutant strain could be due to its lower K_m (79.4 μM) compared with that of the parental enzyme (184 μM). Hence, the mutant enzyme showed a better functionality and thermostability, which will be beneficial for industrial applications.

Key Words: Bilirubin oxidase; *Myrothecium verrucaria*; solid-state fermentation; specific activity; thermostability; UV irradiation

Introduction

Multicopper oxidases (MCOs) are enzymes which utilize four copper ions during the oxidation of their substrates. Laccases, which belong to MCO family, have been well studied due to their applications in the decolorization of dyes, pulp bleaching, polymer synthesis, detoxification and bioremediation (Mayer and Staples, 2002). Bilirubin oxidase also belongs to a sub-group of MCOs and utilizes four copper ions for its catalytic activity. It oxidizes bilirubin to biliverdin (Koikeda et al., 1993) and is used for the detection of serum bilirubin levels and during the diagnosis of jaundice (Doumas et al., 1987). Bilirubin oxidase was first reported to be produced in the submerged cultures of the fungus *Myrothecium verrucaria* MT-1 (Murao and Tanaka, 1981; Tanaka and Murao,1982). Later this enzyme was reported to be produced from the submerged cultures of other strains of *Myrothecium*, i.e. *Myrothecium* sp IMER (Zhang et al., 1982), *M. verrucaria* 3.2190 (Han et al., 2012) and *M. verrucaria* Mv 2.1089 (Guo et al., 1991).

Bilirubin oxidase was also reported to be produced in the submerged cultures of other fungi, *Schizophyllum com-*

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mune K-17 (Matsui et al., 1986a), *Trachyderma tsunodae* K-2593 (Matsui et al., 1986b) and *Penicillium janthinellum* M 5613 (Takahashi et al., 1988). It was also reported to be produced from the submerged culture of a bacterium, *Bacillius licheniformis* B-0891 (Yoshino et al., 1988). In addition, this enzyme activity was produced in the submerged cultures of other microorganisms, *Coprinus micaceus* HU8302, *Trametes hirsuta* HU 8311, *Trametes versicolor* HU 8312, *Coriolus consors* HU 8313, *Pholiota nameko* HU 8321, *Pleurotus ostreatus* HU 8331, *Lenzites styracina* HU 8341 and *Fomitopsis castanea* HU 8351 (Uwajima and Ando, 1987).

Bilirubin oxidases show high activity at neutral pH and are resistant to chloride ions and other chelators, unlike laccases (Mano, 2012). However, there have been only a few reports on the utilization of bilirubin oxidases for health care and environmental applications (Mano, 2012). Hence, bilirubin oxidases need to be explored much more fully. But, above all, native strains produce low levels of bilirubin oxidases and improving their yields will benefit the biotech sector. Some attempts have been made to improve the yields by expressing the bilirubin oxidase of *M. verrucaria* in yeast (Koikeda et al., 1993) and *Pichia pastoris* (Kataoka et al., 2005). Solid-state fermentation was also adopted for improving the yields of bilirubin oxidase from *Myrothecium* sp IMER1 (Liu et al., 2009), and this is the only report available for the production of bilirubin oxidase by solid-state fermentation. However, in these studies, yields were not highly improved even though there was some improvement. In the present study, productivity of bilirubin oxidase has been improved by using a bilirubin-oxidase-producing mutant strain of *M. verrucaria*, and studies were carried out on the functionality and thermostability of the bilirubin oxidase produced by the mutant strain in the solid-state fermentation process.

Materials and Methods

Fungal strain and culture conditions. The culture of *M. verrucaria* (MTCC 2140) was maintained on potato dextrose agar slants containing glucose (1%), agar (1.5%) and 40% potato extract at 4°C. For the production of bilirubin oxidase, the culture was grown in the medium containing wheat flour (1%) and 40% potato extract at pH 7.0 and 30°C for 72 h. The culture broth was filtered to remove the fungal biomass and then centrifuged, and the supernatant was used to estimate the bilirubin oxidase activity.

Enzyme assays. The bilirubin oxidase activity was assayed by adding 1 ml of appropriately diluted enzyme or culture filtrate to 2.0 ml of 0.002% bilirubin dissolved in 0.2 M Tris-HCl buffer (pH 7) and incubating at 37°C for 5 min. The decrease in OD at 440 nm was measured. One unit of enzyme corresponds to the amount of bilirubin oxidase that oxidizes 1 micromole of bilirubin min^{-1} at 37°C and pH 7. Protein concentration was estimated by the Bradford method and the specific activity was calculated as the activity mg^{-1} protein. The reaction was carried out at different pHs (3–9) and temperatures (25–50°C) while determining out the optimum activity of the enzyme.

The thermostability of the enzyme was studied by incubating equal amounts of enzyme (100 U) from the parent and mutant at 60°C, and residual activities were measured at different time intervals and the time ($t_{1/2}$) required to inactivate half of the initial enzyme activity at 60°C was determined. The functionality of bilirubin oxidase produced from parent and mutant strains was checked by incubating an equal amount of enzyme (20 U) with 10 ml of 0.02% bilirubin at 37°C and the OD at 440 nm was recorded at different time intervals (0–10 min). The K_m values of bilirubin oxidase enzyme from the parent and mutant strains were measured by adding 5 U of enzyme to the reaction mixtures containing different concentrations of bilirubin (0.0006–0.0018%), measuring the enzyme activities and plotting Lineweaver-Burk plots.

Strain improvement. A young spore suspension of *M. verrucaria* in sterile water was spread on the agar plates containing glucose (1%) and potato extract (40%) and exposed to UV light (302 nm) for different time intervals (0–60 s) (Ravi-Kumar et al., 2004). The plates were then incubated overnight in the dark at 4°C and thereafter at 30°C for 72 h. White, or fast growing, sectors from the survived colonies were selected and grown on the potato dextrose agar plates containing bilirubin at 30°C for 72 h. The colonies showing a higher zone of clearance compared with the parent were inoculated into the liquid medium containing wheat flour (1%) and 40% potato extract and grown at 30°C for 72 h. The mutant cultures were subcultured for at least five generations, and the ones that consistently overproduced the enzyme compare with the parent strain were selected.

Solid-state fermentation studies. Inoculum (10 ml) of the parent or mutant strain grown in the medium containing wheat flour (1%) and 40% potato extract was added to the 50 g of solid-substrate sterilized in 500 ml Erlenmeyer flasks and mixed thoroughly, and incubated at the required temperature (25–40°C), initial pH (4–9) and humidity (50–100%). At different periods (0–144 h) of fermentation, the fermented substrate was dried at 37°C and extracted with the buffer (0.2 M Tris-HCl, pH 7) and the bilirubin oxidase activity was estimated as described earlier. The activity was expressed per g dry substrate.

Downstream processing. After the solid-state fermentation, 1 kg of wheat bran, fermented by either the parent or mutant strain, was air dried at 37°C and mixed thoroughly with 10 volumes of buffer (0.05 M Tris-HCl, pH 7) at 30°C and 200 rpm for 2 h. The mixture was filtered through a muslin cloth and centrifuged, and the supernatant was concentrated 10 times by ultrafiltration. The concentrated sample was 1:1 diluted with phosphate buffer (10 mM, pH 7.0) and subjected to ammonium sulphate (80%) precipitation, and the precipitate was separated by centrifugation (5000 rpm and 4°C) and dissolved in the required volume of phosphate buffer and dialysed against the same buffer. The sample was loaded on to DEAE-cellulose column equilibrated with the phosphate buffer (10 mM, pH 7.0) and eluted with 0.2–1 M NaCl prepared in the same buffer in an isocratic manner at a flow rate of 1 ml/min.
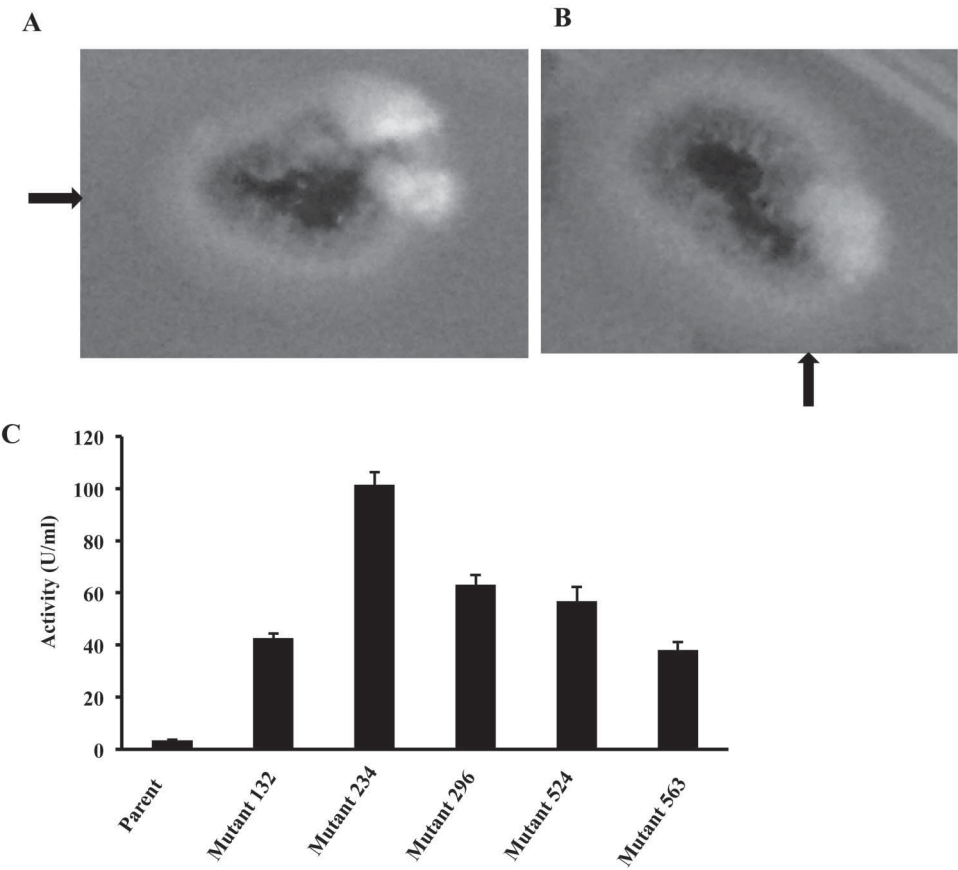
Fig. 1. Mutant colonies of *M. verrucaria* and their bilirubin oxidase productivity.

Mutant colonies (A and B) of *M. verrucaria* showing white, and fast growing, sectors. Arrows point to the fast growing sectors. (C) Bar graph showing the productivity of bilirubin oxidase by the parent and mutant strains of *M. verrucaria*.
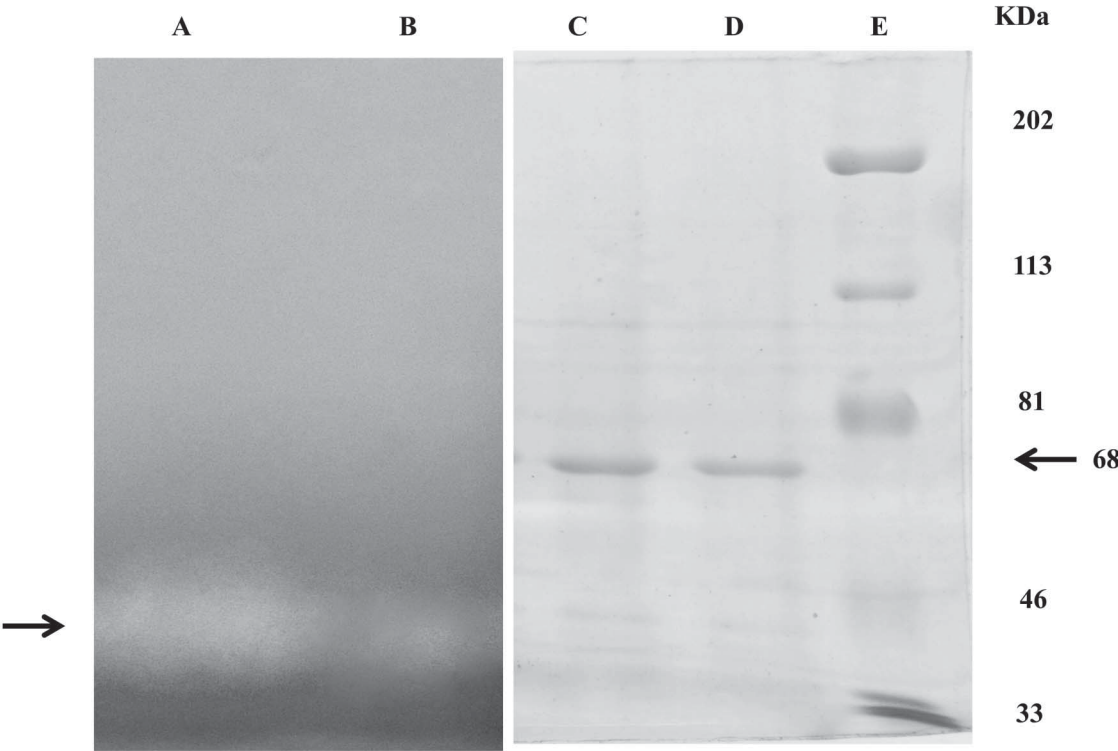

Fig. 2. Polyacrylamide gels showing the activities and molecular masses of bilirubin oxidase produced by the mutant (lanes A and C) and parent (lanes B and D) strains of *M. verrucaria*.

Lanes A and B correspond to the activity staining, and lanes C and D correspond to the SDS-PAGE. Lane E shows the molecular weight markers.
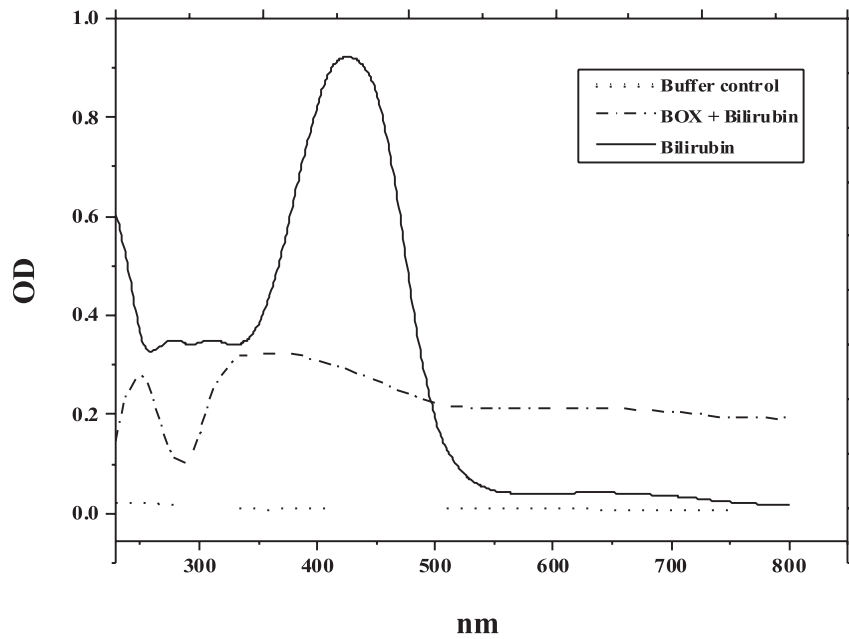
Fig. 3. UV-Vis spectra of bilirubin in the presence and absence of bilirubin oxidase (BOX) purified from the mutant strain.
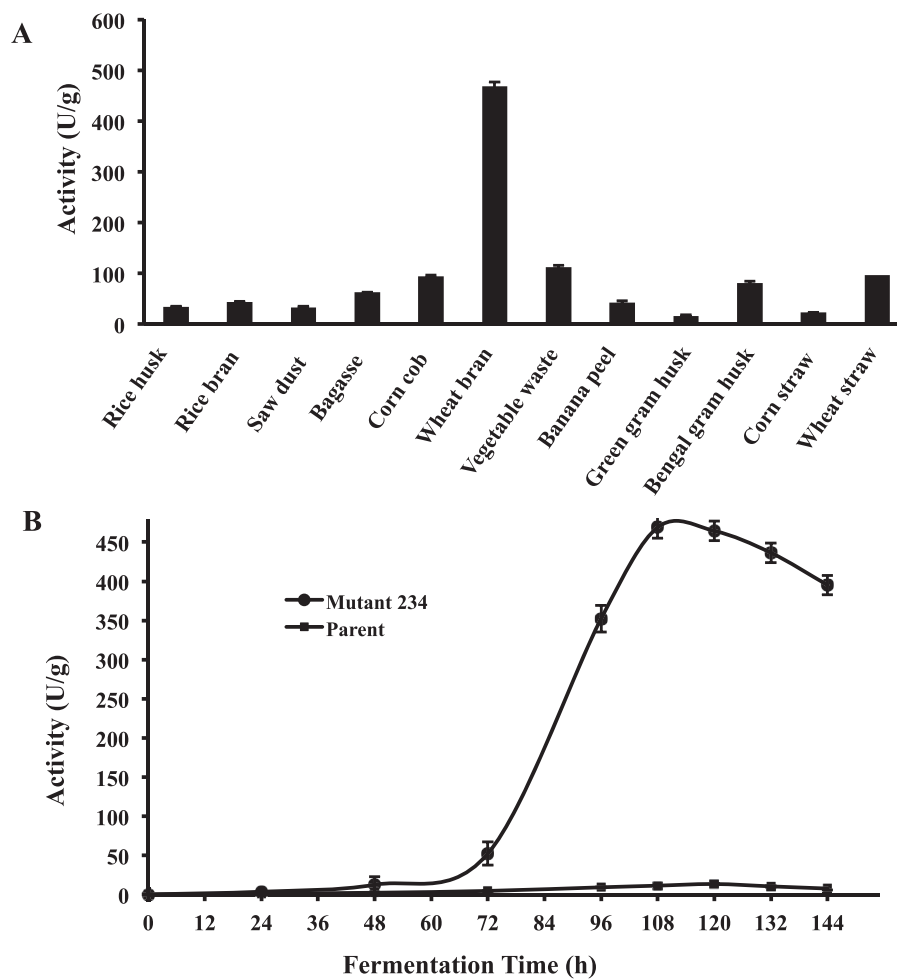

Fig. 4. Production of bilirubin oxidase in the solid-state fermentation.

A. Enzyme productivity by the mutant strain of *M. verrucaria* in different solid substrates. B. Graph showing the production of bilirubin oxidase by the parent and mutant strains in the wheat bran at different periods of fermentation.

Table 1.   Purification of bilirubin oxidase from the wheat bran fermented by parent and mutant strains of *M. verrucaria*.

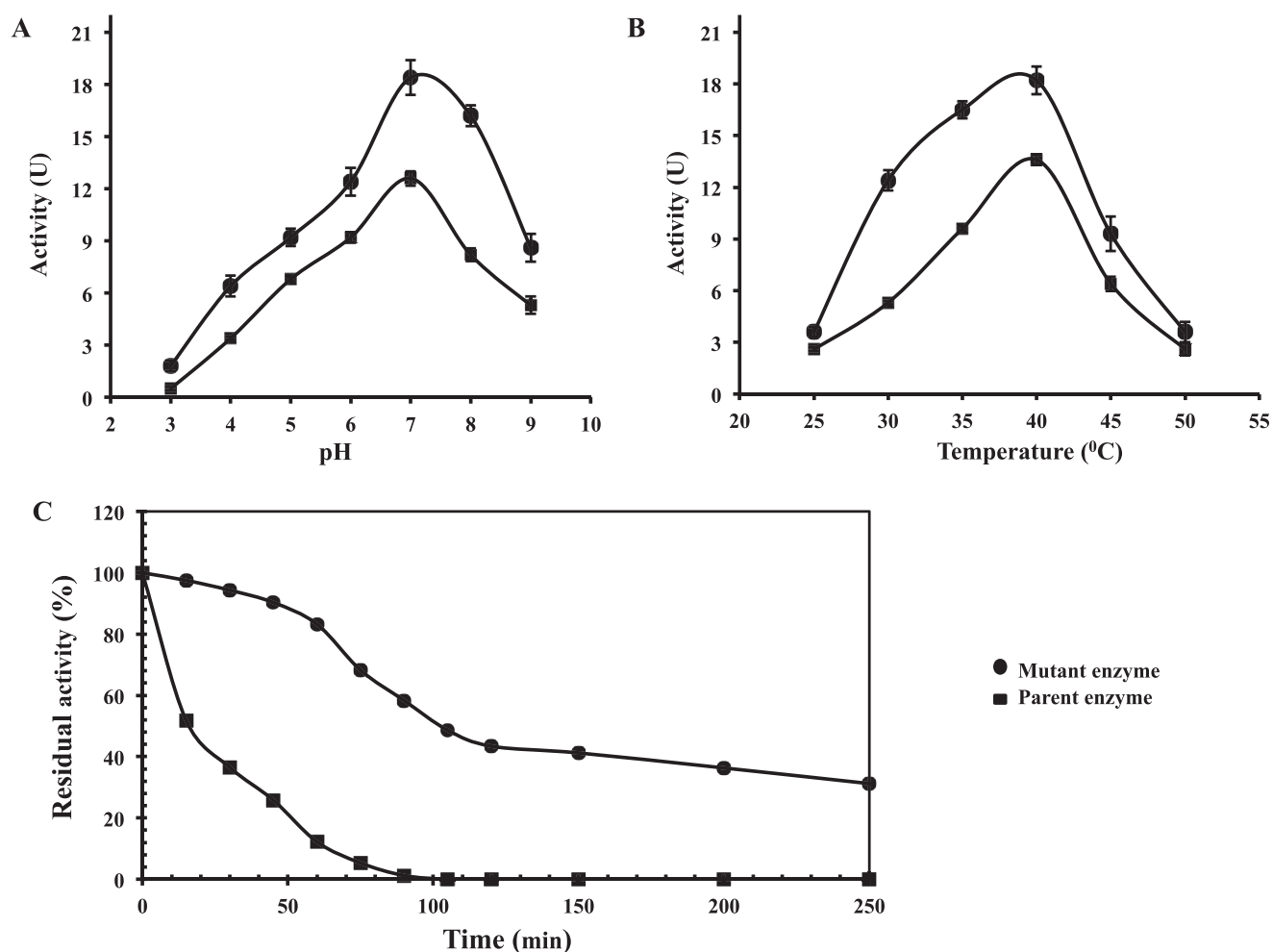| S. No.	Recovery step		Total activity (U)	Specific activity	Recovery (%)
1.	Ultrafiltration	Mutant	405390	33.4	100
		Parent	12951	1.12	100
2.	Precipitation by ammonium sulphate	Mutant	325846	106.5	80.37
		Parent	9853	5.4	76.07
3.	Separation by ion-exchange chromatography (DEAE-cellulose)	Mutant	278540	254.5	68.71
		Parent	6464	9.3	49.91
4.	Separation by gel-exclusion chromatography (Sephadex-G-100)	Mutant	245981	480.16	60.67
		Parent	5536	15.45	42.74

Fig. 5. Graphs showing the optimum conditions and thermostablity of bilirubin oxidases obtained from parent and mutant strains of *M. verrucaria*. Graphs showing the activities of the bilirubin oxidase obtained from the parent and mutant strains of *M. verrucaria* at different pH (A) and temperatures (B). (C) Graph showing the thermostabilities of the bilirubin oxidase obtained from the parent and mutant strains at 60°C.

Major fractions showing bilirubin oxidase activity were pooled together, dialysed against Tris-HCl buffer (0.05 M, pH 7) and concentrated by using lyophilizer (–50°C; Lab conco, Kansas City, USA). The concentrated sample was further purified using a Sephadex-G-100 column equilibrated with Tris-HCl buffer (0.2 M, pH 7) and eluted with the same buffer at a flow rate of 0.8 ml/min. The fractions showing enzyme activity were pooled together and the purity of the enzyme was checked on a 10% SDS-polyacrylamide gel.

Electrophoretic studies. Electrophoretic studies were carried out by using 10% polyacrylamide gels. Purified enzyme (10 μg) from the parent and mutant cultures was separated on the 10% SDS-polyacrylamide gel. The gel was stained with a coomassie brilliant blue R250 solution for 1 h and destained until visualization of clear blue bands. For activity staining, the parent and mutant culture filtrates (1000 ml) were precipitated with 80% ammonium sulphate and the precipitated proteins were dialysed against the 0.05 M Tris-HCl buffer (pH 7). The obtained protein
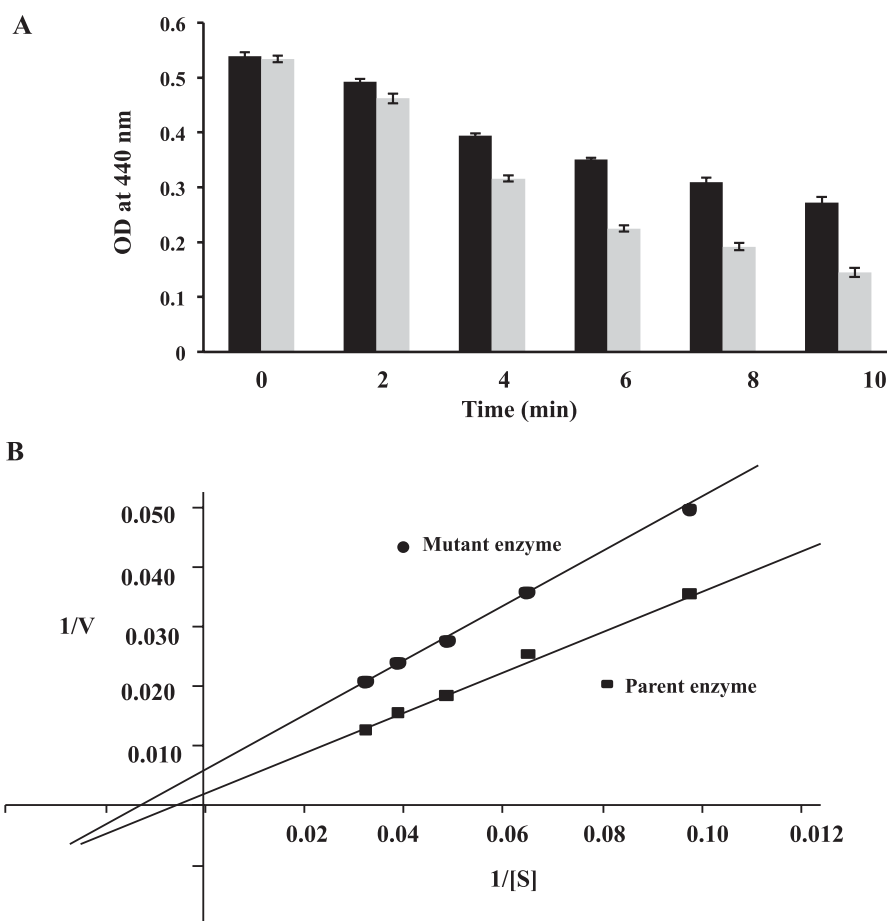
Fig. 6. Graphs showing the functionality and kinetics of bilirubin oxidases obtained from parent and mutant strains of *M. verrucaria*.

A. Bar diagram showing the clearance of bilirubin by the bilirubin oxidase purified from the parent (black bars) and mutant (gray bars) strains of *M. verrucaria* at different periods of time and 37°C. B. Lineweaver-Burk plots showing the K_m of bilirubin oxidase from the parent (184 μM) and mutant (79.4 μM) strains on *M. verrucaria*.

from the parent (10 μg) and the mutant (1 μg) strains was separated on the 10% SDS-polyacrylamide gel without boiling the protein samples. The proteins were then renatured by incubating the gel in the 0.05 M Tris-HCl buffer (pH 7) for 1 h at 37°C. Finally, the gel was incubated in the same buffer containing bilirubin (0.007%) at 37°C until the appearance of zones of clearance against the yellow background.

N-terminal sequencing. Purified enzymes from the parent and mutant strains were separated on the 10% SDS-polyacrylamide gel and transferred to a PVDF membrane and microsequenced.

Results and Discussion

Bilirubin oxidase has potential applications in the health and environmental sectors due to its activity around neutral pH and tolerance to chloride ions and chelators (Mano, 2012). Hence, attempts were made to increase the production of bilirubin oxidase by adopting molecular biological methods (Kataoka et al., 2005; Koikeda et al., 1993) and the maximum production achieved was 5 U/ml (Kataoka et al., 2005). However, UV irradiation was not

explored for improving the strain for bilirubin oxidase production. In this study, *M. verrucaria* (MTCC 2140) was mutated by using UV-rays in order to obtain a strain that overproduces the bilirubin oxidase.

When a young spore suspension of *M. verrucaria* (MTCC 2140) was exposed to UV rays there was an increase in the percentage killing of spores with increase in the time of exposure. Some induced mutations in fungal colonies were manifested as a change in the growth rate, or colour, of sectors. A greater number of colonies containing fast growing and white sectors (Figs. 1A and B) were observed after spores were exposed to UV for 25 s which resulted in 47% of spores being killed. Fast growing and white sectors from 862 colonies were subcultured on potato dextrose agar plates containing bilirubin. The mutant colonies surrounded by a higher zone of clearance, compared with the parent, due to the secreted bilirubin oxidase were inoculated into the enzyme production medium and grown under the optimum conditions. Very high levels of bilirubin oxidase were produced by the few mutant colonies compared with the parent (Fig. 1C). Mutants were subcultured for 5 generations, and the levels of bilirubin oxidase were measured and mutant No. 243 (*M. verrucaria* MTCC 2140-243) was shown to produce higher

levels of the enzyme consistently. This mutant (101 U/ml) showed 28.7 times higher levels of enzyme compared with the parent (3.51 U/ml). Overproduction of the enzyme by the mutant was also seen in the zymogram (Fig. 2, zone of clearance in the lane "A" vs "B") carried out with the substrate, bilirubin. The bilirubin showed a λ_{max} at 440 nm and this peak disappeared in the presence of bilirubin oxidase purified from the mutant strain (Fig. 3). The productivity (101 U/ml) of bilirubin oxidase by the mutant was higher than those reported earlier in submerged cultures (19 U/ml, Murao and Tanaka, 1981; 6.5 U/ml, Matsui et al.,1986a; 1.08 U/ml, Matsui et al., 1986b; 0.05–4.08 U/ml, Uwajima and Ando, 1987; 11.3 U/ml, Takahashi et al., 1988; 200 U/13.8 L, Yoshino et al., 1988; 1.5 U/ml, Guo et al., 1991; 0.33 U/L, Koikeda et al., 1993; 0.7 U/ml, Zhang et al., 1982; 2.7 U/ml, Han et al., 2012). The specific activity of the enzyme in the mutant (62.8) culture was 29.9 times higher than that of the parent (2.1) culture, which is also higher than the recombinant bilirubin oxidase (9.4, Kataoka et al., 2005).

Bilirubin oxidase production by *Myrothecium* sp IMER1 was improved 2 times by adopting solid-state fermentation (Liu et al., 2014) and a productivity of 13.4 U/g was achieved on the bran at pH 4.6, 23°C and 83.5% humidity after 5 days. However, this method was not explored further for improving the production of bilirubin oxidase, even though it was economical. The parent strain (*M. verrucaria* MTCC 2140) produced 12.3 U/g of enzyme under the same conditions, whereas the mutant (*M. verrucaria* MTCC 2140-243) strain produced 410 U/g, which is 33.3 times higher than the parent. The mutant showed the highest productivity at pH 7.0 (data not shown), 30°C (data not shown) and 80% humidity (data not shown). Under the same conditions, the mutant showed the best activity on the wheat bran (Fig. 4A). Mutant-produced maximum amount (468 U/g) of enzyme at 108 h of fermentation and productivity declined afterwards (Fig. 4B) under the above optimum conditions, whereas, in the case of the parent, the highest production (13.1 U/g) was at 120 h (Fig. 4B). The specific activities of the enzyme produced during the solid-state fermentation of the parent (1.12 after 108 h), and the mutant (33.4 after 108 h), strains were lower than that produced in submerged fermentation. However, the enzyme produced by the mutant strain showed a higher specific activity compared to the enzyme produced by the parent and the recombinant enzyme (Kataoka et al., 2005). The mutant showed 35.6 times higher enzyme production than the parent under the optimum conditions, in addition to a reduced fermentation time. Also, the production of bilirubin oxidase, by using the currently developed mutant strain and conditions, is better than the previously reported solid-state fermentation process (Liu et al., 2014). Also, the relative enzyme productivity by the mutant strain compared with the parent was higher in the solid-state fermentation (35.6 times) than submerged fermentation (28.8 times). These results suggest that the mutant strain shows a better productivity of bilirubin oxidase in the solid-state fermentation.

Purification of the enzyme from both the strains was carried out with dry fermented wheat bran (1 kg) by adopting ultra filtration, ammonium sulphate precipitation, DEAE-ion exchange chromatography and gel-exclusion chromatography (Table 1). The purified bilirubin oxidase from the fermented wheat bran of the parent and mutant strains showed a specific activity of 15.4 and 480, respectively. Enzyme from both the strains moved at 68 KDa on the SDS-polyacrylamide gel (Fig. 2, lanes C and D) suggesting no change in the molecular mass of the enzyme in the mutant due to UV irradiation. Enzymes moved below the 68 KDa in the native gel (Fig. 2, lanes A and B) which could be due to the globular structure in the native state. Bilirubin oxidase from both the strains showed an optimum pH of 7 (Fig. 5A) and temperature of 40°C (Fig. 5B). However, the time ($t_{1/2}$) required to inactivate half of the initial enzyme activity (100 U) was much higher in the case of the enzyme obtained from the mutant (105 min) compared with that from the parent (16 min) at 60°C (Fig. 5C). This $t_{1/2}$ is higher than 90 min obtained for recombinant bilirubin oxidase (Kataoka et al., 2005). The improved thermostability of the enzyme from the mutant strain could be due to the point mutations induced during the UV irradiation, since there was no change in the mass of the enzyme compared with that obtained from the parent (Fig. 2, lanes C and D). Also, there was no change in the N-terminal amino acid sequence (VAQISP) of the mutant enzyme compared with the parent, suggesting the induction of point mutations in the other regions of the protein. This sequence matched with the N-terminal amino acid sequence of bilirubin oxidase from *Myrothecium verrucaria* MT-1 (Koikeda et al., 1993).

The bilirubin oxidase of mutant strain degraded the bilirubin faster than the enzyme obtained from the parent (Fig. 6A, compare gray bars with corresponding black bars) after the incubation of equal levels of bilirubin with equal units of enzyme from both the strains under the same conditions. Faster activity of the bilirubin oxidase obtained from the mutant strain could be due to its lower K_m (79.4 μM) compared with that of bilirubin oxidase of the parent (184 μM) (Fig. 6B). These results suggest the better functionality and thermostability for the enzyme produced by the mutant strain which will be beneficial for industrial applications.

Conflict of Interest

The authors declare that there is no conflict of interest.

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