

Isolation of a laccase with HIV-1 reverse transcriptase inhibitory activity from fresh fruiting bodies of the *Lentinus edodes* (Shiitake mushroom)

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Received 28 July 2010; revised 03 January 2011

A laccase with a molecular mass of 67 kDa and inhibitory activity toward HIV-1 reverse transcriptase ($IC_{50} = 7.5 \mu\text{M}$) was isolated from fresh fruiting bodies of the *Lentinus edodes* (Shiitake mushroom). Its characteristics were compared with those of laccases from cultured mushroom mycelia reported earlier. The laccase was unadsorbed on DEAE-cellulose, Affi-gel blue gel and CM-cellulose, but was adsorbed on Con A-Sepharose. About 50-fold purification was achieved with a 19.2% yield of the enzyme. The activity of the enzyme increased steadily from 20°C to 70°C. The activity disappeared after exposure to the boiling temperature for 10 min. Its optimal pH was 4 and very little enzyme activity remained at and above pH 10. The laccase inhibited HIV-1 reverse transcriptase with an IC_{50} of 7.5 μM , but did not demonstrate any antifungal or anti-proliferative activity.

Keywords: *Lentinus edodes*, Shiitake mushroom, laccase, HIV-1 reverse transcriptase inhibitory activity

Lentinus edodes (Shiitake mushroom) is a common edible mushroom, available in the fresh as well as in the dried state. Many compounds with important biological activities have been isolated from the mushroom. These include the polysaccharide lentinan with antitumor and immunomodulating activities¹, a lectin with hemagglutinating activity², proteins with ribonucleolytic activity³, and the antifungal protein lentin⁴. Laccases (EC 1.10.3.2) are lignin-degrading enzymes⁵⁻⁶ which find applications in biosensors, pulping, textile dyes and detoxification of polluted water⁷⁻¹¹. They have been purified from the cultured mycelia of various mushrooms^{7,12-24}. Recently, many new applications are reported for the laccases, such as biocatalysts for industrial effluents, bioremediation, colour and phenolic removal and hair dyeing²⁵⁻²⁷.

In the present study, a laccase has been isolated from the fruiting bodies of the *L. edodes* and its characteristics have been compared with laccases isolated from cultured mushroom mycelia^{28,29}.

Materials and Methods

Isolation of laccase

An aqueous extract was prepared by homogenizing the fresh fruiting bodies (1.5 g) of *Lentinus edodes* in

distilled water (3 ml/g) using a Waring blender. Tris-HCl buffer (1 M, pH 7.4) was added to the supernatant obtained by centrifuging the homogenate until the concentration of Tris reached 10 mM. The supernatant was loaded on a 5 × 20 cm column of DEAE-cellulose (Sigma). Unadsorbed proteins (fraction D1) were eluted with 10 mM Tris-HCl buffer (pH 7.4). Adsorbed proteins (fraction D2) were desorbed with the same buffer to which 0.8 M NaCl had been added. Fraction D1 was next chromatographed on a 2.5 × 20 cm column of Affi-gel blue gel (Bio-Rad) in 10 mM Tris-HCl buffer (pH 7.4). After elution of unadsorbed proteins (fraction B1), adsorbed proteins (fraction B2) were eluted with 10 mM Tris-HCl buffer (pH 7.4) containing 1.2 M NaCl.

Fraction B1 was applied on a 2.5 × 20 cm column of CM-cellulose (Sigma) in 10 mM NH_4OAc buffer (pH 4.5). Unadsorbed proteins were collected as fraction CM1. Adsorbed proteins were desorbed with 10 mM NH_4OAc containing 1 M NaCl and collected as fraction CM2. Fraction CM1 was subjected to affinity chromatography on a 2.5 × 20 cm column of Con A-Sepharose (GE Healthcare) in 50 mM Tris-HCl (pH 7.4) containing 0.5 M NaCl, 10 mM CaCl_2 and 10 mM MgCl_2 . After elution of unadsorbed proteins (fraction Con A1), adsorbed proteins (fraction Con A2) were eluted with the afore-mentioned 50 mM Tris-HCl buffer containing 0.4 M methyl- α -D-glucopyranoside. Fraction Con A2 was further purified on a Superdex 75

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HR 10/30 column (GE Healthcare) in 0.2 M NH_4HCO_3 buffer (pH 8.5) using an AKTA purifier (GE Healthcare). The first peak represented the purified laccase³⁰.

Determination of laccase activity

Laccase activity was assayed by measuring the oxidation of 2,2'-azinobis [3-ethylbenzothiazolone-6-sulfonic acid] diammonium salt (ABTS). A modification of the method of Shin and Lee²¹ was used. An aliquot of enzyme solution was incubated in 1.3 ml of 67 mM sodium acetate buffer (pH 4.5) containing 1.54 mM ABTS at 30°C. One unit of enzyme activity was defined as the amount of enzyme required to produce an absorbance increase at 420 nm of one per min per ml of reaction mixture under the aforementioned condition²⁸. The activity of laccase towards other substrates including ABTS, N,N-dimethyl-1,4-phenylenediamine, hydroquinone, catechol, 2-methylcatechol, pyrogallol, and tyrosine was also tested.

Molecular mass determination by SDS-PAGE and by FPLC-gel filtration

SDS-PAGE was carried out accordance with the procedure of Laemmli and Favre³¹ using a 12% resolving gel and a 5% stacking gel. At the end of electrophoresis, the gel was stained with Coomassie brilliant blue. FPLC-gel filtration was carried out using a Superdex 75 column which had been calibrated with molecular mass standards (GE Healthcare), including phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa).

Temperature and pH optima

To estimate the temperature optima of laccase, the above-mentioned assays were applied at temperatures ranging from 20 to 80°C. The optimum pH was estimated by monitoring the residual enzyme activity at pH ranging from 3 to 9.

Analysis of N-terminal amino acid sequence

Amino acid sequence analysis was carried out using an HP G1000A Edman degradation unit and an HP1000 HPLC system³².

Assay for HIV-1 reverse transcriptase inhibitory activity

The assay for HIV reverse transcriptase inhibitory activity was carried out since some mushroom have laccases demonstrated this activity^{33,34}. The assay was

done according to instructions supplied with the assay kit from Boehringer Mannheim (Germany). This assay was conducted in view of previous reports of HIV-1 reverse transcriptase inhibitory activity in some mushroom proteins^{30,35}. The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, starting from the template/primer hybrid poly (A) oligo (dT) 15. The digoxigenin and biotin-labeled nucleotides in an optimized ratio were incorporated into one of the same DNA molecule, which was freshly synthesized by the reverse transcriptase (RT). The detection and quantification of synthesized DNA as a parameter for RT activity allows a sandwich ELISA protocol. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been pre-coated with streptavidin. In the next step, an antibody to digoxigenin conjugated to peroxidase binds to the digoxigenin-labeled DNA. In the final step, the peroxidase substrate was added. The peroxidase catalyzes the cleavage of the substrates, producing a colored reaction product. The absorbance of the samples at 405 nm was determined using a microtiter plate (ELISA) reader and was directly correlated to the level of RT activity. A fixed amount (4-6 ng) of recombinant HIV-1 reverse transcriptase was used. The inhibitory activity of isolated protein was calculated as percent inhibition, as compared to a control without the protein³⁶.

Results

Purification of laccase

Fraction D1 unadsorbed on DEAE-cellulose, but not the adsorbed fraction D2, possessed the laccase activity. Fraction B1 derived from fraction D1 by affinity chromatography on Affi-gel blue gel exhibited significantly higher laccase activity than the adsorbed fraction B2. Ion-exchange chromatography of fraction B1 on CM-cellulose yielded an unadsorbed fraction CM1 which contained the bulk of laccase activity (Table 1). Fraction CM1 was resolved on Con A-Sepharose into a large unadsorbed fraction (Con A1) without much laccase activity and a much smaller adsorbed fraction (Con A2) in which laccase activity was enriched (Fig. 1, Table 1). Fraction Con A2 was resolved on Superdex 75 into a higher peak S1 with laccase activity and a lower peak S2 with little activity (Fig. 2, Table 1). Peak S1 represented the purified laccase.

Determination of molecular mass and N-terminal sequence

The purified laccase appeared as a single band with a molecular mass of 67 kDa in SDS-PAGE (Fig. 3).

Table 1—Yields and laccase activities of aqueous extract and various chromatographic fractions derived from 1.5 kg fresh fruiting bodies of *L. edodes*

Chromatographic fractions	Protein yield (mg)	Specific laccase activity (U/mg)	Total activity (U)	Recovery of activity (%)	Purification fold
Extract	4810	0.73	3511.3	100	1
D1	1430	1.59	2273.7	64.8	2.2
D2	1820	<0.1	-	-	-
B1	792	2.14	1694.9	48.3	2.9
B2	293	0.47	137.7	3.9	0.6
CM1	398	3.36	1337.3	38.1	4.6
CM2	162	<.01	-	-	-
ConA1	237	0.32	75.8	2.2	0.4
ConA2	45.3	23.2	1051.0	29.9	31.8
S1	18.7	36.72	686.7	19.6	50.3
S2	11.5	2.5	28.8	0.8	3.4

Laccase activity was determined at 30°C and pH 4.

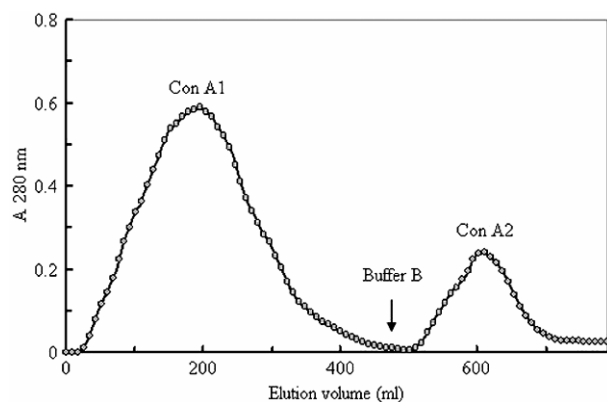


Fig. 1—Affinity chromatography on Con A-Sepharose [Sample: fraction of fruiting body extract unadsorbed on DEAE-cellulose and subsequently unadsorbed on Affi-gel blue gel. Column dimensions: 2.5 × 20 cm. Buffer: 50 mM Tris-HCl buffer containing 0.5 M NaCl, 10 mM CaCl₂ and 10 mM MgCl₂ (pH 7.4). Fraction size: 7 ml. Buffer containing 0.4 M α -methyl-D-glucopyranoside was used to elute the adsorbed proteins]

It showed little resemblance to other mushroom laccases in N-terminal sequence (Table 2).

Temperature and pH dependence of laccase activity

The activity of laccase showed a temperature-dependent rise until the temperature reached 70°C. At 80°C, the activity started to fall (Fig. 4). The optimal pH was around 4 and at and above pH 6, there was a marked decline in activity (Fig. 5). The laccase inhibited HIV-1 reverse transcriptase with an IC₅₀ of 7.5 μ M.

Substrate specificity of laccase

The enzyme oxidized a variety of substrates, including polyphenolic substrates (hydroquinone, pyrogallol, catechol), methoxy-substituted phenols

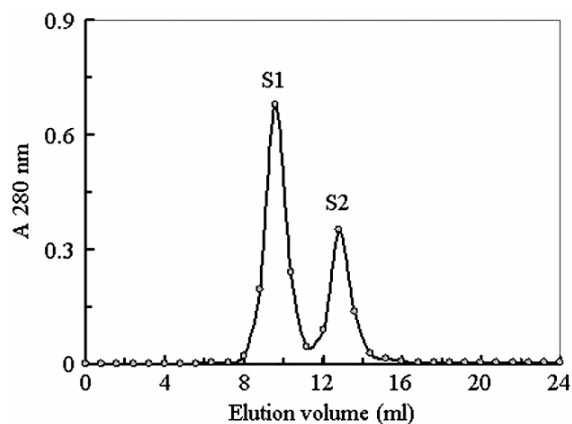


Fig. 2—Gel-filtration by fast protein liquid chromatography on a Superdex 75 HR 10/30 column [Sample: peak Con A2 from Con A-Sepharose column. Buffer: 0.2 M NH₄HCO₃ (pH 8.5). Flow rate: 0.4 ml/min. Fraction size: 0.8 ml]

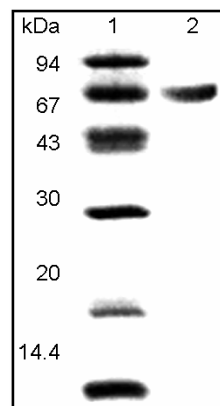
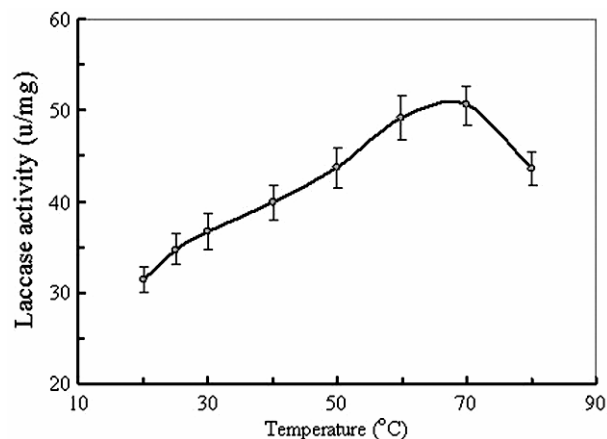
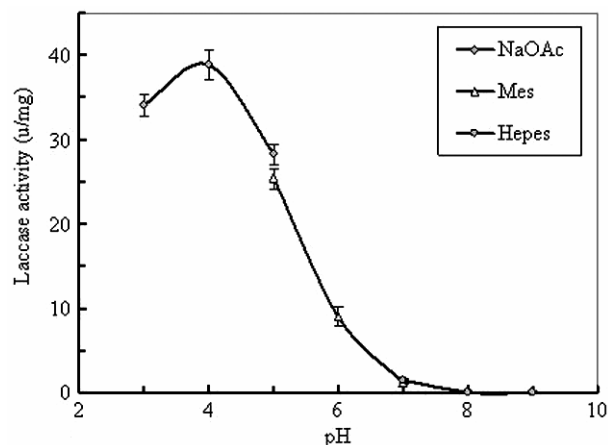


Fig. 3—SDS-PAGE results [Left lane: molecular mass markers (GE Healthcare), from top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α -lactalbumin (14.4 kDa). Right lane: *L. edodes* laccase]

Fig. 4—Effect of temperature on laccase activity of *L. edodes* laccaseFig. 5—Effect of pH on laccase activity of *L. edodes* laccaseTable 2—N-terminal sequence comparison between *L. edodes* laccase and other laccases

Mushroom laccase	N-terminal sequence
<i>Agaricus bisporus</i> laccase I ²¹	KTRTFDFDLV
<i>A. bisporus</i> laccase II ²¹	DTKTFNFDLV
<i>Albatrella dispansus</i> laccase ⁴⁸	AQPPNYHYN
Basidiomycetes PM1 laccase ¹⁵	SIGPV.ADLTI
<i>Cantherellus cibarius</i> laccase ⁴⁷	GCCNCGHA
<i>Cariporiopsis subvermispora</i> laccase ²¹	AIGPVTDLFI
<i>Coriolus hirsutus</i> laccase ²¹	AIGPT.ADLTI
<i>Ganoderma lucidum</i> laccase ⁴⁹	GQNGDAVP
<i>Hericium erinaceum</i> laccase ⁴³	AVDDDAEQIP
<i>Lentinus edodes</i> laccase (this study)	AGTSHFADL
<i>L. edodes</i> L54 laccase (mycelial) ³⁹	YGQTVSENLFIVN
<i>L. edodes</i> SR-1 laccase (mycelial) ²⁸	AIGPVTDLHIVN
<i>Pleurotus eryngii</i> laccase I (fruiting body) ³⁴	AVGPVLGPDA
<i>P. eryngii</i> laccase I (mycelial) ²¹	AXKKLDFHII
<i>P. eryngii</i> laccase II (mycelial) ²¹	ATKKLDFHII
<i>Phlebia radiata</i> laccase ¹⁴	SIGPVTDFHI
<i>Pycnoporus cinnabarius</i> laccase ²¹	AIGPV.ADLTI
<i>Trametes versicolor</i> laccase I ²¹	AIGPVASLVV
<i>T. versicolor</i> laccase II ²¹	GIGPV.ADLTI
<i>T. versicolor</i> laccase III ²¹	GIGPV.ADLTD
<i>Tricholoma giganteum</i> laccase ³³	DDPQQAVIDD

Space created to maximize sequence similarity. Identical corresponding amino acids are underscored.

(2-methylcatechol), aromatic diamine (N,N-dimethyl-1,4-phenylenediamine) and the non-phenolic heterocyclic compound ABTS. Compared with the ABTS, the enzyme showed 60% activity towards N,N-dimethyl-1,4-phenylenediamine, about 10% activity towards pyrogallol, 2-methylcatechol and hydroquinone, minimal activity (8%) towards catechol, and no activity towards tyrosine (Table 3).

Comparison with other mushroom laccases

L. edodes laccase resembled laccases from *Albatrella dispansus*, *Cantherellus cibarius* and

Table 3—Activities of laccase from *L. edodes* toward various substrates

Substrate	Wavelength (nm)	Relative activity (%)
N,N-Dimethyl-1,4-phenylenediamine	515	60.2
ABTS	420	100.0
Hydroquinone	248	11.7
2-Methylcatechol	436	12.5
Pyrogallol	450	13.0
Catechol	450	8.2
Tyrosine	280	0.0

Ganoderma lucidum in chromatographic behavior on ion-exchangers, *A. dispansus*, *C. cibarius*, and *Tricholoma giganteum* in optimum pH, *A. dispansus*, *G. lucidum*, *Pleurotus eryngii*, and *T. giganteum* in optimum temperature, and *G. lucidum*, *Hericium erinaceum*, *P. eryngii* and *T. giganteum* in HIV-1 reverse transcriptase inhibitory activity (Table 4).

Discussion

Laccases are widely present in the fungi and are responsible for many physiological functions, such as fungal spore development, melanization³⁷ and degradation of complex organic substances including lignin or humic matter³⁸. Due to their broad substrate range, laccases are finding many new applications, thus fungi are good source to find new laccases for industrial applications.

A 72 kDa laccase has been purified from mycelia of *L. edodes* SR-1 using chromatography on butyl-650 M, Toyopearl D-650 M, Superose 12, mono Q and phenyl Saperose²⁸. Another 53-58 kDa laccase has been isolated from *L. edodes* fruiting bodies by employing chromatography on Toyopearl CM-650 M,

Table 4—Comparison of biochemical characteristics and activities of laccases from *L. edodes* and other mushroom laccases

	<i>Albatrella dispansus</i> ⁴⁸	<i>Cantherellus cibarius</i> ⁴⁷	<i>Ganoderma lucidum</i> ⁴⁹	<i>Hericium erinaceum</i> ⁴³	<i>L. edodes</i> (This study)	<i>L. edodes</i> # (lacI/lacII) ^{28,29}	<i>Pleurotus eryngii</i> ³⁴	<i>Pleurotus florida</i> ⁴⁵	<i>P. ostreatus</i> ⁷	<i>Pleurotus pulmonarius</i> ⁴⁶	<i>Tricholoma giganteum</i> ³³
Molecular mass (kDa)	62	92	75	63	67	72/58	34	77	75	46	43
Chromatographic behavior on											
(i) DEAE-ion exchange	UN	UN	UN	AD	UN	AD/-	AD	AD	AD	AD	UN
(ii) Q-ion exchange	-	-	-	AD	-	AD/-	AD	-	AD	-	-
(iii) CM-ion exchange	-	-	-	UN	UN	- /AD	UN	-	-	-	AD
(iv) Affigel blue gel	UN	UN	UN	-	UN	-/-	-	-	-	-	AD
(v) Con A-Sepharose	AD	AD	AD	-	AD	-	-	-	-	-	-
Optimum pH	4	4	3-5	5	4	4/3	-	-	Alkaline range	4.0-5.5	4
Optimum temperature (°C)	70	50	70	50	70	40/40	70	-	50	50	70
HIV-1 reverse transcriptase inhibitory activity (IC ₅₀)	No activity	-	1.2 µM	9.5 µM	7.5 µM	-/-	2.2 µM	-	-	-	2.2 µM

AD, adsorbed, UN, unadsorbed; -, not determined or not attempted

Superdex 75 and phenyl sapperose²⁹. The characterization, molecular cloning and differential expression analysis of a laccase gene (lac 1) from *L. edodes* L54 encoding a 526-amino acid laccase has also been reported³⁹. However, no biological activities of *L. edodes* laccase have been described before. The laccases isolated by Nagai *et al*^{28,29} differ from the laccase encoded by Zhao and Kwan³⁹ and the laccase isolated in the present study in N-terminal sequence. The laccase reported in this study differed in molecular mass, optimum temperature and optimum pH from the laccases isolated by Nagai *et al*^{28,29}, indicating that different strains of the *L. edodes* were used in the different studies. *L. edodes* laccase fermentation in submerged and solid-state and heterologous expression has been studied recently^{40,41}.

Shiitake mushroom laccase manifested an N-terminal sequence with only slight similarity to that of *Coriolus hirsutus* laccase and was different from the sequences of *L. edodes* laccases reported earlier^{28,29,39}. The molecular mass of shiitake mushroom laccase (67 kDa) was close to that of *Coriolus hirsutus* (73 kDa), and intermediate between those (58 and 72 kDa) of *L. edodes* laccases isolated by Nagai *et al*^{28,29}. The laccases also differed in other aspects. While shiitake mushroom laccase was unadsorbed on DEAE-cellulose, *C. hirsutus* and the *L. edodes* laccases^{28,29}

were adsorbed on DEAE-ion exchanger²¹. The pH optima of laccase from Shiitake mushroom and *C. hirsutus* was 4 and 2-3, respectively. The activity of shiitake mushroom laccase increased until the temperature reached 70°C and considerable activity was present at 80°C. On the other hand, *L. edodes* laccases of Nagai *et al*^{28,29} had an optimum temperature at 40°C and *C. hirsutus* laccase displayed a optimum temperature at 45°C beyond which the activity declined²¹.

The adsorption of shiitake mushroom laccase on Con A-Sepharose suggested that it a glycoprotein, like previously laccases isolated from *L. edodes* by Nagai *et al*^{28,29}. The laccases isolated from *L. edodes* by Nagai *et al*^{28,29} were derived from cultured mycelia, while fruiting bodies were employed for the isolation of shiitake mushroom laccase. Shiitake mushroom laccase was unadsorbed on a variety of chromatographic media, including DEAE-cellulose, CM-cellulose and Affi-gel blue gel. Hence Con A-Sepharose contributed significantly to the purification of the laccase. In contrast, *L. edodes* laccases of Nagai *et al*²⁸⁻²⁹ are adsorbed on DEAE- and CM-ion exchangers.

The shiitake mushroom laccase inhibited HIV-1 reverse transcriptase like other mushroom proteins, such as lectins⁴², ubiquitin-like proteins³⁰, ribosome

inactivating proteins^{32,35}, and antifungal proteins³². Laccases from some mushroom species display this activity^{33,34,43}. The inhibitory potency of shiitake mushroom laccase was similar to some of the natural products³⁶. The laccase isolated in this study was devoid of anti-proliferative and antifungal activities, although some mushroom lectins, antifungal proteins and ribonucleases have shown anti-proliferative activity^{30,42,44} and mushroom proteins impede fungal growth³².

In conclusion, shiitake mushroom laccase was found to be different from those of previously reported *L. edodes* laccases^{28,29,39} in N-terminal sequence, molecular mass, optimum pH, optimum temperature and chromatographic behavior on cation and anion exchangers. The laccase inhibited HIV-1 reverse transcriptase with an IC₅₀ of 7.5 µM, but was devoid of any antifungal or anti-proliferative activity.

Acknowledgments

This work was financially supported by National Grants of China (2006BAD07A01 and nyhyzx07-008) and a direct grant from Medicine Panel of Research Committee, CUHK.

References

- Chihara G, Hamuro J, Maeda Y, Arai Y & Fukuoka F (1970) *Cancer Res* 30, 2776-2781
- Wang H X, Ng T B & Ooi VEC (1999) *Int J Biochem Cell Biol* 31, 595-599
- Kobayashi H, Inokuchi N, Koyama T, Watanabe H, Iwama M, Ohgi K & Irie M (1992) *Biosci Biotechnol Biochem* 56, 2003-2010
- Ngai P H & Ng T B (2003) *Life Sci* 73, 3363-3374
- Evans C S, Dutton M V, Guillen F & Veness R G (1994) *FEMS Microbiol Rev* 13, 235-240
- Thurston C F (1994) *Microbiol* 140, 19-26
- Palmieri G, Giardina P, Marzullo L, Desiderio B, Nitti G, Cannio R & Sannia G (1993) *Appl Microbiol Biotechnol* 39, 632-636
- Brenna O & Bianchi E (1994) *Biotechn Lett* 16, 35-40
- Reid I D & Paice M G (1994) *FEMS Microbiol Rev* 13, 369-376
- Ghindilis A L, Makower A, Bier F F & Scheller F W (1995) *Anal Methods Instrum* 2, 129-132
- Martirani L, Giardina P, Marzullo L & Sannia G (1996) *Water Res* 30, 1914-1918
- Pickard M A & Westlake D W (1970) *Can J Biochem* 48, 1351-1358
- Leonowicz A & Malinowska M (1982) *Acta Biochim Pol* 29, 219-226
- Saloheimo M, Niku-Paavola M L & Knowles J K (1991) *J Gen Microbiol* 137, 1537-1544
- Coll P M, Fernandez-Abalos J M, Villanueva J R, Santamaria R & Perez P (1993) *Appl Environ Microbiol* 59, 2607-2613
- Perry C R, Matcham S E, Wood D A & Thurston C F (1993) *J Gen Microbiol* 139, 171-178
- Giardina P, Palmieri G, Scaloni A, Fontanella B, Faraco V, Cennamo G & Sannia G (1999) *Biochem J* 341 (Pt 3), 655-663
- Sethuraman A, Akin D E & Eriksson K E (1999) *Appl Microbiol Biotechnol* 52, 689-697
- Cambria M, Cambria A, Ragusa S & Rizzarelli E (2000) *Protein Expr Purif* 18, 141-147
- Dedeyan B, Klionowska A, Tagger S, Tron T, Iacazio G, Gil G & Le Petit J (2000) *Appl Environ Microbiol* 66, 925-929
- Shin K S & Lee Y J (2000) *Arch Biochem Biophys* 384, 109-115
- Fukuda T, Uchida H, Takashima Y, Uwajima T, Kawabata T & Suzuki M (2001) *Biochem Biophys Res Commun* 284, 704-706
- Garzillo A M, Colao M C, Buonocore V, Oliva R, Falcigno L, Saviano M, Santoro AM, Zappala R, Bonomo RP, Bianco C *et al* (2001) *J Protein Chem* 20, 191-201
- Tinoco R, Pickard M A & Vazquez-Duhalt R (2001) *Lett Appl Microbiol* 32, 331-335
- Montazer M, Dadashian F, Hemmatinejad N & Farhoudi K (2009) *Appl Biochem Biotechnol* 158, 685-693
- Teixeira R S, Pereira P M & Ferreira-Leitao V S (2010) *Enzyme Res* 2010, 905896 (Published online)
- Zhou M F, Yuan X Z, Zhong H, Liu Z F, Li H, Jiang L L & Zeng G M (2010) *Appl Biochem Biotechnol* (This article is Epub, ahead of print)
- Nagai M, Sato T, Watanabe H, Saito K, Kawata M & Enei H (2002) *Appl Microbiol Biotechnol* 60, 327-335
- Nagai M, Kawata M, Watanabe H, Ogawa M, Saito K, Takesawa T, Kanda K & Sato T (2003) *Microbiology* 149, 2455-2462
- Wang H X & Ng T B (2000) *Biochem Biophys Res Commun* 276, 587-593
- Laemmli U K & Favre M (1973) *J Mol Biol* 80, 575-599
- Lam S K & Ng T B (2001) *Arch Biochem Biophys* 393, 271-280
- Wang H X & Ng T B (2004) *Biochem Biophys Res Commun* 315, 450-454
- Wang H X & Ng T B (2006) *Appl Microbiol Biotechnol* 69, 521-525
- Wang H & Ng T B (2001) *Life Sci* 68, 2151-2158
- Collins R A, Ng T B, Fong W P, Wan C C & Yeung H W (1997) *Life Sci* 60, PL345-351
- Fowler Z L, Baron C M, Panepinto J C & Koffas M A (2010) *Yeast* 28, 181-188
- Claus H (2003) *Arch Microbiol* 179, 145-150
- Zhao J & Kwan H S (1999) *Appl Environ Microbiol* 65, 4908-4913
- Elisashvili V, Penninckx M, Kachlishvili E, Tsiklauri N, Metreveli E, Kharziani T & Kvesitadze G (2008) *Bioresour Technol* 99, 457-462
- Sakamoto Y, Nakade K, Yano A, Nakagawa Y, Hirano T, Irie T, Watanabe H, Nagai M & Sato T (2008) *Appl Microbiol Biotechnol* 79, 971-980

- 42 Wang H X & Ng T B (2001) *Planta Med* 67, 669-672
- 43 Wang H X & Ng T B (2004) *Biochem Biophys Res Commun* 322, 17-21
- 44 Wang H X, Ng T B & Ooi V E (1998) *Biochem Mol Biol Int* 44, 135-141
- 45 Das N, Chakraborty T K & Mukherjee M (2000) *Folia Microbiol (Praha)* 45, 447-451
- 46 Marques De Souza C G & Peralta R M (2003) *J Basic Microbiol* 43, 278-286
- 47 Ng T B & Wang H X (2004) *Biochem Biophys Res Commun* 313, 37-41
- 48 Wang H X & Ng T B (2004) *Biochem Biophys Res Commun* 319, 381-385
- 49 Joo S S, Ryu I W, Park J K, Yoo Y M, Lee D H, Hwang K W, Choi H T, Lim C J, Lee do I & Kim K (2008) *Mol Cells* 25, 112-118