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Original article

Elevated levels of laccase synthesis by *Pleurotus pulmonarius* BPSM10 and its potential as a dye decolorizing agent

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ABSTRACT

Laccases (EC 1.10.3.2) are a class of multi-copper oxidases that have industrial value. In the present study, forty-five isolates of wild mushrooms were screened for laccase production. Eight of the isolates exhibited exploitable levels of substrate oxidation capacity. Isolate BPSM10 exhibited the highest laccase activity of 103.50 U/ml. Internal Transcribed Spacer (ITS) rRNA gene sequencing was used to identify BPSM10 as *Pleurotus pulmonarius*. The response of BPSM10 to two nutritional media supplemented with various inducers was characterized and the results indicated that Malt Extract Broth (MEB) supplemented with Xylidine increased laccase production by 2.8× (349.5 U/ml) relative to the control (122 U/ml), while Potato Dextrose Broth (PDB) supplemented with xylidine increased laccase production by 1.9× (286 U/ml). BPSM10 has the ability to decolorize seven synthetic dyes in a liquid medium. Maximum decolorization was observed of malachite green (MG); exhibiting 68.6% decolorization at 100 mg/L. Fourier-transform infrared spectroscopy (FTIR) was employed to confirm the decolorization capacity. The present study indicates that *P. pulmonarius* BPSM10 has the potential to be used as a potent alternative biosorbent for the removal of synthetic dyes from aqueous solutions, especially in the detoxification of polluted water.

1. Introduction

Laccase (EC 1.10.3.2; benzenediol: oxygen oxidoreductase) is one of the oxidative enzymes responsible for the degradation of lignin (Atalla et al., 2010). Laccases are widespread among higher plants, fungi and bacteria. In particular, white rot fungi are the most important and predominant producer of this enzyme. The biodegradation properties of laccases highlight its potential use in several industrial processes, including biopulping, textile dye bleaching, degradation of aromatic pollutants, detoxification of polluted water, and its potential use as a biosensor (Zhuo et al., 2011). Various groups of synthetic dyes including azo, triphenylmethane, anthraquinone, as well as heterocyclic and phthalocyanine dyes, are extremely stable and highly resistant to degradation by microbial agents. Decolorization of dyes by conventional methods, such as coagulation, flocculation, sorption, electrochemical decomposition, or oxidative degradation have limitations due to their high cost and the secondary pollution that results from the use of these physicochemical treatments (Si and Cui, 2013). Several microorganisms have been examined for their potential use in the decolorization of synthetic dyes. Specifically, white rot fungi have been found to be one the most suitable microorganisms to use for degradation of a broad range of environmental pollutants, including highly recalcitrant dyes (Nerud et al., 2004).

The white rot fungus, *Pleurotus pulmonarius,* which typically grows on standing dead trees and fallen logs, is well-known for its extracellular secretion of laccase when cultured under submerged or solid-state conditions using wheat bran as a substrate. Its ability to decolorize textile dyes in both systems has also been

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documented (dos Santos et al., 2013). In the present study, a newly isolated strain of a wild mushroom, identified as *P. pulmonarius* BPSM10, was obtained from a protected forest area and examined for its ability to produce laccase. In addition, the ability of BPSM10 to decolorize synthetic dyes, including dyes from azo and triphenylmethane groups, was also examined. The results indicate that *P. pulmonarius* BPSM10 has the potential to serve as an alternative biosorbent for the removal of synthetic dyes from aqueous environments.

2. Materials and methods

2.1. Chemicals, dyes and organisms

2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), Guaiacol (o-Methoxyphenol), naphthol blue black, orange G, malachite green, victoria blue B, phenol red, congo red, and Coomassie brilliant blue were purchased from HiMedia, Mumbai, Maharashtra, India. All chemicals used were of analytical grade. A variety of wild mushrooms were collected from the Dampa Tiger Reserve (DTR) and Murlen National Park (MNP) in India (Fig. S1) and mycelial cultures were grown and maintained on Potato Dextrose Agar (PDA) at 28 ± 2 °C. The fungal cultures were maintained by periodic sub-culturing on PDA slants at 28 ± 2 °C and storage at 4 °C (Zhuo et al., 2011).

2.2. Screening and quantification of laccase

Qualitative screening for laccase activity was performed using acetate buffered substrate media (Kiiskinen et al., 2004) maintained at pH 5.5 and supplemented with 0.1% v/v Guaiacol (o-Methoxyphenol). Production of laccase was indicated by the appearance of a reddish-brown colour on the culture plate. Isolates which exhibited laccase activity were grown in Potato Dextrose Broth (PDB) amended with 0.025% v/v of 100 mM $CuSO_4$ as a laccase inducer. Other inducing agents were also evaluated. Cultures were grown for 6 days at 28 ± 2 °C with constant agitation at 100 rpm in a shaking incubator. Cell free supernatants, considered as a crude enzyme preparation, were obtained by centrifuging the culture media at 10,000 rpm for 10 min.

Quantitative estimation of laccase was determined in a reaction mixture containing 3 mM 2, 2′ - Azino-bis-(3-ethylbenzothiazo line-6-sulfonic acid) diammonium salt (ABTS) and 100 mM citrate buffer (pH 5.5) (Park and Park, 2014). Laccase activity was assessed by measuring the increase in the absorbance of the reaction product at 420 nm (ε = 36, 000 M^{-1} cm^{-1}). One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute at 30 °C.

2.3. Taxonomic identification of isolate BPSM10

BPSM10, the fungal isolate with the highest laccase activity, was taxonomically identified by amplification of the ITS rRNA gene as previously described (Lallawmsanga et al., 2016). The obtained sequence was queried against ITS sequences available in GenBank, using ClustalW, and a dendrogram was constructed to establish taxonomic position (Thompson et al., 1997). The BPSM10 isolate has been deposited in the Indian Type Culture Collection (ITCC), New Delhi under accession number ITCC-8215.

2.4. Protein estimation

Aliquots of cell free extract of strain BPSM10 at an appropriate dilution were used for the estimation of soluble protein content (Lowry et al., 1951). Bovine serum albumin was used to construct a standard curve. The different concentrations of BSA used were 0.1, 0.2, 0.4, 0.6, 0.8, 0.9, 2.0 and 6.0 mg/ml.

2.5. Dye decolorization and UV–Vis analysis

Seven azo and triphenylmethane dyes (Naphthol Blue Black, Orange G, Malachite Green (MG), Victoria Blue B, Phenol Red, Congo Red, and Coomassie Brilliant Blue) were used to evaluate the ability of BPSM10 to decolorize dye solutions. The decolorization assay was performed in 100 ml Erlenmeyer flasks containing 50 ml Malt Extract Broth (MEB) supplemented with 0.1% v/v xylidine and 10 ml of crude laccase enzyme extract obtained from BPSM10. Several different concentrations of dye (Table 2) were added to the described media.

Decolorizing ability was determined spectrophotometrically as the relative decrease in absorbance over time in three independent samples. Absorbance was measured at or near the wavelength maximum for each dye at each time interval. Percentage decolorization was calculated using the formula established by Ayed et al. (2010) and the decolorization efficiency was calculated as follows:

$$\text{Decolorization}(\%) = \frac{A_i - A_t}{A_i} \times 100$$

where A_i is the initial absorbance of dye, and A_t is the absorbance of dye after decolorization.

The supernatants (300 μl) of the degraded samples were scanned in the range of 300–800 nm using an UV–vis (Multiscan™ GO, ThermoScientific, MA, US) spectrophotometer to observe the spectral shifts caused by the biotransformation of dye.

2.6. FT-IR (Fourier transform infrared spectroscopy) analysis

Pellets from control (100 mg of MG per L of media) and tested samples (100 mg/L of MG treated with crude laccase of BPSM10) were dried and mixed with FT-IR grade potassium bromide (1:20; 0.02 of sample with KBr at a final weight of 0.4 g). Samples were ground in an agate pestle and mortar and processed through a hydraulic press. The absorbance Fourier transform infrared (FT-IR) spectra of the samples were recorded using a JASCO FT-IR 6800 within a scanning range of 400–4000 cm^{-1} and 64 scans per second (Olukanni et al., 2006).

2.7. Statistical analysis

The presented data represent the mean ± SE of three replicates (n = 3). Significant differences between treatments were determined using a Fisher's LSD multiple range test at a 5% level of probability.

3. Results

3.1. Qualitative and Quantitative laccase activity

A total of 45 isolates collected from the Dampa Tiger Reserve and Murlen National Park exhibited varying levels of laccase activity (Table S1). Among them, eight isolates (BPSM01, 02, 07, 08, 10, 16, 20, and 24) that exhibited laccase substrate oxidation capacity above 1.5 mm were selected for further quantification. The laccase quantification assay (Table 1) of the selected 8 isolates revealed that isolate BPSM10 had the highest laccase activity (103.50 Uml^{-1}), followed by BPSM16 (93.42 Uml^{-1}), and BPSM20 (80.12 Uml^{-1}). Isolate BPSM10 was selected for further study based on having the highest laccase activity.

Table 1
Laccase enzyme activity of eight selected wild mushroom isolates.

Sl. No.	Name of the isolates	Laccase (U/ml)
BPSM01	*Schizophyllum commune*	66.32
BPSM02	*Trametes hirsuta*	48.11
BPSM07	*Trametes hirsuta*	44.55
BPSM08	*Trametes hirsuta*	40.32
BPSM10	*Pleurotus pulmonarius*	**103.50**
BPSM16	*Bjerkandera adusta*	93.42
BPSM20	*Polyporus* sp.	80.12
BPSM24	*Trametes elegans*	22.04

3.2. Phylogenetic analysis of isolate BPSM10

Partial amplification of the ITS gene of BPSM10 was conducted and the obtained sequence (645 bp) was identified as *Pleurotus pulmonarius* (NCBI GenBank accession number KJ865840). The ITS sequence of BPSM10 exhibited 100% sequence similarity with *Pleurotus pulmonarius* isolate WM10T. The constructed phylogenetic tree revealed that BPSM10 is most closely related to the *Pleurotus pulmonarius* strain WM10T, followed by *Pleurotus pulmonarius* voucher HMAS86396, and *Pleurotus pulmonarius* isolate UD 25/08 with a bootstrap support value of 66% (Fig. 1A).

3.3. Effect of different inducers on laccase activity

The addition of xylidine in MEB resulted in maximum induction of laccase activity, relative to the control, other inducers, and other growth media (Fig. S2). A 2.8× increase (349.5 U ml^{-1}) in laccase activity, relative to the control (122 U ml^{-1}), while a 1.9× (286 U ml^{-1}) increase was observed when xylidine was added to PDB growth medium.

In contrast, the use of $CuSO_4$ as an inducer resulted in higher laccase activity in PDB (196 U ml^{-1}) than in MEB (146 U ml^{-1}). Guaiacol also stimulated laccase activity in PDB by 1.08× (156 U ml^{-1}) but had a lesser inducing effect (145.5 U ml^{-1}).

Maximum laccase activity was observed on the 8th day of incubation in MEB supplemented with xylidine, followed by the 10th day of incubation in PDB with xylidine (Fig. 1B). Maximum extracellular secretion of protein (43.46 mg/ml), however, was observed on the 6th day of incubation in PDB with xylidine, followed by the 6th day of incubation in MEB supplemented with xylidine (Fig. 2A). Interestingly, on the day of maximum laccase production, the protein concentration in MEB supplemented with xylidine was 35.33 mg/ml and 28.98 mg/ml in PDB with xylidine.

3.4. Decolorization assays

Crude laccase enzyme exhibited variable levels of ability to decolorize the seven selected synthetic dyes belonging to the azo and triphenylmethane groups. Among the dyes studied, Malachite Green was decolorized within the first hour of co-incubation with enzyme, exhibiting a decolorization percentage of 68.61% at a dye concentration of 100 mg/L. Congo Red (500 mg/L), Naphthol Blue Black (100 mg/L), Orange G (500 mg/L), and Coomassie Brilliant Blue (300 mg/L) were decolorized at a rate of 48.59%, 46.2%,

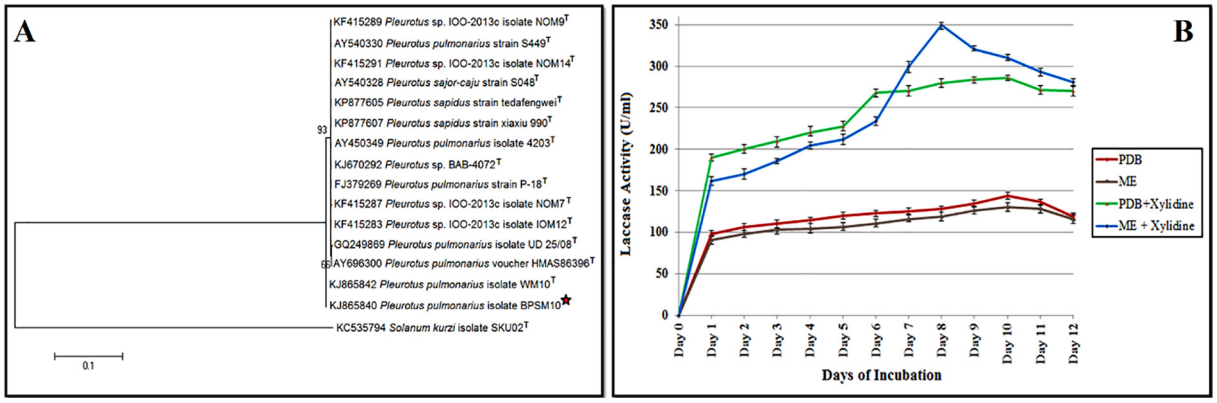

Fig. 1. (A) Phylogenetic tree constructed based on Kimura-2 model based on Maximum Likelihood Method under 1000 bootstrap replicates. (B) Effect of xylidine on laccase activity of *P. pulmonarius* BPSM10.

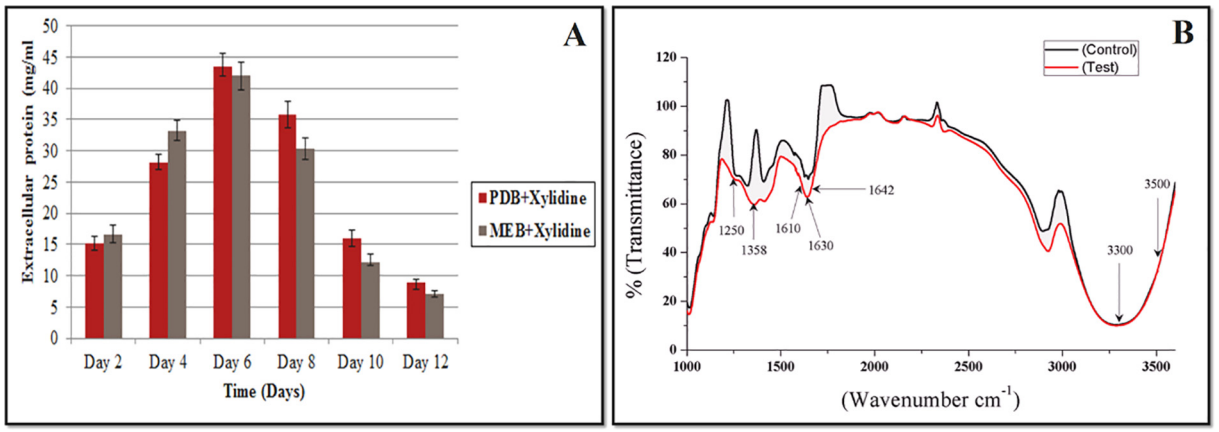

Fig. 2. (A) Extracellular protein content of *P. pulmonarius* BPSM10. (B) FT-IR spectra of Malachite Green treated with *P. pulmonarius* BPSM10 against the control sample.

Table 2
Decolorization of synthetic dyes by crude laccase enzyme obtained from *P. pulmonarius* strain BPSM10.

Dye	λ_{max}	Concentration dye used (in %)	1 hr	6 hrs	12 hrs	24 hrs	36 hrs	48 hrs
Naphthol Blue Black	681	0.01 (100 mg/L)	46.20 ± 0.24	51.02 ± 0.64	**55.26 ± 0.67**	55.10 ± 0.70	55.00 ± 0.97	55.09 ± 0.10
Orange G	550	0.05 (500 mg/L)	42.09 ± 0.84	47.31 ± 0.96	53.56 ± 0.73	56.21 ± 0.40	**58.58 ± 0.51**	58.08 ± 0.81
Malachite Green	670	0.01 (100 mg/L)	**68.61 ± 0.64**	**68.41 ± 0.33**	68.21 ± 0.54	68.28 ± 0.51	68.31 ± 0.70	68.01 ± 0.86
Victoria Blue B	680	0.05 (500 mg/L)	6.87 ± 0.32	10.45 ± 0.58	12.12 ± 0.27	**16.03 ± 0.42**	15.83 ± 0.69	15.58 ± 0.90
Phenol Red	525	0.1 (100 mg/L)	7.88±0.82	13.26 ± 0.82	18.10 ± 0.82	20.25 ± 0.31	**22.22 ± 0.82**	21.89 ± 0.53
Congo Red	430	0.05 (500 mg/L)	48.59±0.34	53.71 ± 0.47	57.62 ± 0.17	**61.14 ± 0.36**	61.07 ± 0.47	61.10 ± 0.34
Coomassie Brilliant Blue	660	0.03 (300 mg/L)	20.49±0.13	21.04 ± 0.16	26.81 ± 0.11	31.19 ± 0.25	**33.12 ± 0.23**	32.28 ± 1.17

Table 3
Functional groups of decolorized malachite green and its respective wavenumbers.

Wave number (cm^{-1})	Functional Group	Corresponding compound
1610–1630	s—N=N—s	Azo linkages of aromatic structure
1642	C=C, C=O	Conjugation groups (carboxylic acid, ketone, ester, or aldehyde)
3300	OH	Azo linkage
3500	OH	Azo linkage
1340–1250	NH_2	Ammonia group

42.09%, and 20.49%, respectively (Table 2). Almost 50% of these latter dyes were decolorized within 6 h of incubation with crude laccase extract. With the exception for Malachite Green, the effect of crude laccase on the decolorization of triphenylmethane dyes (Victoria Blue B, Phenol Red, and Coomassie Brilliant Blue) was the least effective with the highest decolorization percentages reported at around 24–36 h; with a decolorization percentage of 16%, 22%, and 33%, respectively.

3.5. FT-IR analysis

FT-IR was used to help identify functional groups in the treated and untreated dyes, which would provide information on the transformed products resulting from the enzymatic degradation of the dyes. The FT-IR spectra and assigned functional groups are presented in Fig. 2B and Table 3. The spectral changes and reduction in the intensity of peaks at wave numbers 1610–1630 and 1358 cm^{-1} in the treated sample relative to the untreated control indicates the breakdown of azo groups and its corresponding bonds, including conjugates that may have attached to the dye. More importantly, a reduction in the intensity at 1642 cm^{-1} in the treated sample relative to the control suggests that conjugate molecules, such as the carboxylic group in the aromatic ring, may have been reduced or transformed. Moreover, the absence of new peaks at 3300 and 3500 cm^{-1} in the treated sample may be due to the absence of azo bonds.

4. Discussion

Ecosystems can be negatively impacted by toxic/polluting organic chemicals of anthropogenic origin. Textile effluents are known to disturb aquatic environments as textile industries generate large quantities of wastewater contaminated with organic dyes and solvents. Organic effluents produced by dye industries are of environmental concern because they are often discharged into water bodies that are also used for agricultural purposes (Sathishkumar et al., 2013). Arora and Sharma (2010) reported that the decolorization of synthetic dyes and effluents generated by the textile industry by physical or chemical methods are not completely effective; thus, enzyme-based decolorization methods are in greater demand.

The main objective of screening different fungi is to identify isolates that produce biological compounds which have industrial applications; including the enzymatic decolorization of dyes (Ang et al., 2011). The formation of unique colours or clear zones in culture plates due to the production of ligninolytic enzymes is evidence of multi-enzymatic activity which could be utilized in a variety of bioremediation processes (Sathishkumar et al., 2013; Machado et al., 2005).

The ITS gene sequence and phylogenetic analysis of BPSM10 resulted in its identification as *Pleurotus pulmonarius* and indicated that it was most closely-related to *P. pulmonarius* isolate WM10T; followed by *P. pulmonarius* voucher HMAS86396, and *P. pulmonarius* isolate UD 25/08 with a bootstrap support value of 66%. This finding is similar to the results obtained by Adebayo et al. (2012) who reported that strain *P. pulmonarius* LAU 09, clustered within a *P. pulmonarius* complex with a bootstrap value of 77%. The LAU 09 isolate was also reported to have high antimicrobial and anti-inflammatory activity.

The ligninolytic activity of white rot fungi is dependent on several factors that vary in their impact among different fungal species. Various compounds like copper sulphate, guaiacol, veratryl alcohol, and xylidine have been widely used to enhance laccase production by fungi (Usha et al., 2014). The current study focused on the enhancement of laccase production by xylidine, guaiacol, and copper sulphate based on previous reports by Papinutti and Forchiassin (2000), Marques and Peralta (2003), and Kuhar et al. (2015). Dhakar and Pandey (2013) on their work on *Trametes hirsuta* (MTCC 1139), reported laccase production and activity in the high pH 5, and activity range from pH 5–7. Atalla et al. (2013), showed that laccase from *Trematosphaeria mangrovei* that was purified showed enzyme activity optima at pH 4 even. Among the laccase reported from *Pleurotus* genera as such Krishna-Prasad et al. (2005), maximal laccase production from *Pleurotus ostreatus* at a pH value of 5.5.

In our study, we examined the ability of crude laccase enzyme extract to decolorize several structurally variable dyes from the azo and triphenylmethane groups of dyes. These two groups represent the two main chemical dye groups used within the industry. Results indicated that *P. pulmonarius* BPSM10 has the ability to decolorize high concentrations of the selected azo and triphenylmethane dyes. Svobodová et al. (2008) reported that crude enzyme extracts of *Irpex lacteus* decolorized Naphthol Blue Black efficiently at a concentration of 150 mg/L. Eichlerova et al. (2006) reported that extracts of *Pleurotus calyptratus* and *Ischnoderma resinosum* degraded Orange G; another environmental pollutant.

Different concentrations of dyes and different organisms have been used in previous studies with varying results. *Lentinula* (*lentinus*) *edodes* and *Trametes pubescens* were examined for the ability to decolorize Congo Red (Si and Cui, 2013; Si et al., 2013). Their results indicated that the highest percentage of decolorization by *Lentinula edodes* was observed at a dye concentration of 200 mg/L (Boer et al., 2004). The percentage of malachite green decolorization in previous reports was lower compared to the results obtained in the current study (Table S1). Dye decolorization can be used as a screening tool to identify promising strains with

potential industrial use that can be further characterized in greater detail. The current study demonstrated the high decolorization efficiency and potential use of *P. pulmonarius* BPSM10 laccase in the treatment of synthetic dye effluents.

FT-IR is an important technology that can be used to detect compounds that represent transformed azo linkages. It is used to identify the presence of azo groups of —N=N—s in aromatic structures and —N=N— stretching in the dye molecules. It is clear that the peaks ranging from 1610 to 1630 to 1312 cm^{-1} are due to the presence of s—N=N—s azo linkages in the aromatic structure and —N=N— stretching in α substituted compounds in the dye molecule, respectively. These peaks are altered by the incubation of the dye with the crude enzyme extract obtained from *Pleurotus pulmonarius* BPSM10. The peak observed at 1642 cm^{-1} is due to the conjugation of C=C and C=O groups; confirming the presence of a carbonyl group in a carboxylic acid, ketone, ester, or conjugated aldehyde attached to an aromatic ring (Maier et al., 2004). The lack of peaks between 3300 and 3500 cm^{-1} is due to the absence of azo bonds and OH groups in positions related to the azo linkages. The absence of peaks between 1250 and 1340 cm^{-1}, which are related to NH_2, indicate the transformation of the NH_2 group to N_2 or NH_3 or into biomass (Kanagaraj et al., 2015).

Authors contributions

L conducted all of the entire experiments and prepared a draft manuscript. AKP, VVL, and IKM supported L in conducting the experiments and in preparing the manuscript. AA reviewed the statistical analysis. SU and BPS reviewed the experiments, and checked and approved the final data. AH, SU, EFA, and BPS contributed to the writing of the initial manuscript and subsequent revisions. All authors read and approved the manuscript prior to submission.

Competing interests

There are no competing interests to declare.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sjbs.2018.10.006.

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