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A rapid method to detect and estimate the activity of the enzyme, alcohol oxidase by the use of two chemical complexes - acetylacetone (3,5-diacetyl-1,4-dihydrolutidine) and acetylacetanilide (3,5-di-*N*-phenylacetyl-1,4-dihydrolutidine)

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ABSTRACT

A rapid and sensitive method has been devised in order to detect and estimate the synthesis of the enzyme alcohol oxidase (AOX) by fungi, by way of the use of two chemical complexes, namely, acetylacetone (3,5-diacetyl-1,4-dihydrolutidine) and acetylacetanilide (3,5-di-*N*-phenylacetyl-1,4-dihydrolutidine). This method involves the use of the AOX enzyme that could specifically oxidize methanol, giving rise to equimolar equivalents each of formaldehyde (HCHO) and hydrogen peroxide (H_2O_2) as the end products. Further, the formaldehyde, thus produced was allowed to interact with the neutral solutions of acetylacetone and the ammonium salt, gradually developing a yellow color, owing to the synthesis and release of 3,5-diacetyl-1,4-dihydrolutidine (yellow product; λ = 420 nm; $\lambda_{ex/em}$ = 390/470 nm) and the product, so generated was quantified spectrophotometrically by measureing its absorbance at 412 nm. In another set up, the amount of formaldehyde produced as a sequel to the oxidation of methanol by the AOX enzyme was determined by allowing it to react with the acetylacetanilide reagent, after which the volume of the fluorescent product - 3,5-di-*N*-phenylacetyl-1,4-dihydrolutidine (colorless product; $\lambda_{ex/em}$ = 390/470 nm) that was generated was estimated by measuring its emission at 460 nm (excitation wavelength at 360 nm) in a spectrophotometer. Of the various substrates tested, a commercial source of the AOX enzyme appreciably oxidizes methanol, thereby generating formaldehyde, and further reacts with acetylacetone, to give rise to a bright yellow complex, displaying a maximum activity of 1402 U/mL. Determination of the AOX activity by the use of acetylacetone and acetylacetanilide could serve as a viable alternative to the conventional alcohol oxidase-peroxidase-2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (AOX-POD-ABTS) based method. In view of this, this method appears to be invaluable for application at the various food, pharmaceutical, fuel, biosensor, biorefinery, biopolymer, biomaterial, platform chemical, and biodiesel industries.

1. Introduction

Alcohol oxidase (AOX; EC 1.1.3.13; methanol oxidase) is known to mediate the events that are associated with the oxidation process of methanol by the involvement of the molecular oxygen as a terminal electron acceptor, wherein an equivalent volume of both hydrogen peroxide (H_2O_2) and formaldehyde (HCHO) is generated as the end products. Besides, this enzyme is also capable of oxidizing a wide range of the lower chain alcoholic substrates (C1-C8 carbon compounds), at a relative velocity to the order of: methanol > ethanol > n-propanol > n-butanol, leading to the generation of hydrogen peroxide and formaldehyde (HCHO) (Goswami et al., 2013). The AOX enzyme offers an advantage in that it does not oxidize the branched chain alcohols - the C_2-aldehydes or certain higher species, or ketones, nor the organic acids.

A brown-rot fungus is believed to secrete certain accessory enzymes, including the alcohol oxidase, glyoxal oxidase, besides the aryl-alcohol oxidase during the decomposition process of the biomass. The AOX enzymes released during that process act as the hydrogen peroxide (H_2O_2) generating ones and are believed to mediate the reactions, by means of a non-enzymatic mode, thereby generating the free hydroxyl radicals (·OH) which are known to play a key role in the modification of the celluloses, hemicelluloses and lignin from the degenerating biomass. In the biomass mineralization process, the initial step is believed

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to be the lignin degradation that paves the way for a demethylation event, which strips off the *O*-methyl ($-OCH_3$) groups from an aryl methoxyl group (methoxyl substitution) thereby, primarily producing methanol, with the participation of the lignin specific *O*-demethylases or certain ligninolytic enzymes (Filley et al., 2000; Filley et al., 2002; Bashtan-Kandybovich et al., 2012; Kohler et al., 2017). Interestingly, some recent studies carried out on the genome sequence of the wood-rot fungi, particularly the brown-rot fungi, suggested that the genes encoded for the alcohol, involved in the functioning of the oxidase enzyme have been overexpressed; this enzyme oxidizes the methanol that is generated during the lignin demethylation process (Martinez et al., 2009, 2011). Several investigators successfully identified and purified the AOX enzyme from the yeast and the various other filamentous fungi grown in the presence of methanol as a substrate (Kato et al., 1976). A cursory glance at the recent developments on this subject would reveal that considerable interest has been evinced in the AOX enzymes. Enzymologists have studied the novel alcohol oxidase and the glycolate oxidase activity in the *Ochrobactrum* sp. AIU 033 (Yamada et al., 2014; Abdelaziz et al., 2016). In this study, we have successfully devised a rapid and sensitive method for the spectroscopic determination of the AOX enzymes by the action against acetylacetone or acetylacetanilide. The currently available method is based on an AOX-peroxidase-ABTS enzyme complex system, which is difficult to analyze with either the lignin based or the phenolic substrates. Their presence is detectable in the fermented materials, mainly because the laccases or peroxidases utilize them as a substrate. Therefore, these methods making use of acetylacetone and acetylacetanilide could very well replace the currently available methods, owing to the availability of the biological materials which provide a rich source of the laccases or peroxidases which catalyze a simultaneous oxidization of ABTS. The following reactions occur:

$$\text{Methanol} + O_2 \xrightarrow{\text{Alcohol Oxidase}} \text{Formaldehyde} + H_2O_2$$

$$H_2O_2 + \text{ABTS} \xrightarrow{\text{Peroxidase}} 2\,H_2O + \text{ABTS (oxidized)}$$

We successfully carried out an AOX-catalyzed oxidation of methanol for generating a formaldehyde based product (Fig. 1) in the first place, by employing the neutral solutions of acetylacetone and ammonium salt and allowing them to react with formaldehyde (HCHO), which gradually develops a yellow color, owing to the synthesis of diacetyldihydrolutidine. Secondly, by means of using the formaldehyde that was produced as a product of the AOX catalyzed oxidation of methanol, which was determined by allowing it to react with an acetylacetanilide reagent, and the fluorescence emitted from the product formed was measured at 460 nm (excitation wavelength at 360 nm) in a spectrofluorometer. The standard curves of the formaldehyde and methanol oxidation-generated formaldehyde by the mediation of the AOX enzyme using acetylacetone and acetylacetanilide were plotted. In order to validate the method that was developed, it was tested against a set of the commercial AOX enzymes by carrying out the oxidation of the following alcohols in the laboratory: methanol, ethanol, isopropanol, 2-methoxy ethanol, 1-propanol and n-butanol, etc., essentially by making use of acetylacetone and acetylacetanilide. The acetylacetone and acetylacetanilide methods were checked for their ability to detect the AOX activity with the Kraft lignin (KL) and the veratryl alcohol raised cultures of the various lignin-demethylating fungi. The methodology adopted in the present study, for a qualitative identification of the enzyme, the alcohol oxidase that is naturally secreted during the microbial fermentation process and the lignin degradation events have been furnished below under the Materials and Methods.

2. Materials and methods

2.1. Chemicals

Kraft lignin (KL; FPI Innovation, Canada), the alcohol oxidase enzyme (AOX; EC 1.1.3.13 solution from the *Pichia pastoris* buffered aqueous solution contained 10–40 units/mg protein (biuret) A2404 (Sigma-Aldrich), lignin-like model compounds (LMCs) (guaiacol (2-methoxy phenol), 2,6-dimethoxyphenol; 2-hydroxy-3-methoxybenzaldehyde (*o*-vanillin); 3,4-dimethoxybenzaldehyde; 3,4-dimethoxybenzyl alcohol; 4-hydroxy-3-methoxycinnamic acid (ferulic acid); 4-hydroxy-3-methoxycinnamaldehyde; 4-hydroxy-3-methoxybenzoic acid), acetylacetone, and acetylacetanilide. All the other analytical grade chemicals were purchased from the Sigma-Aldrich, Canada.

2.2. Assay for the alcohol oxidase enzyme by the acetylacetone method - the formaldehyde approach

In order to detect the AOX activity, the culture filtrate of the test fungi, reared on the KL and LMCs as the substrates was used as a source of the crude AOX enzyme. The reaction mixture included (1 mL); 100 μL of the enzyme, 100 μL of the substrate (100 mM Methanol), 150 μL of the Citrate Phosphate buffer with pH 3.0 and a 100 μL of acetyl acetone (0.02 M 2,4-pentanedione in 2 M ammonium acetate and 0.05 M acetic acid) in 650 μL of water. The AOX reaction was initiated by adding a 100 μL of the crude enzyme and the same was next incubated with methanol, mixed gently and was vortexed and incubated at 25 °C for 15 min., followed by the addition of a 100 μL of acetylacetone. Next, the contents were mixed thoroughly and subsequently let stand at 60 °C for 15 min. The lignin demethylated samples released methanol, which was allowed to be oxidized in the presence of the commercial alcohol oxidase, which released a μmol equivalent of formaldehyde (HCHO) along with hydrogen peroxide (H_2O_2) as the products. Approximately neutral solutions of acetylacetone and the ammonium salt were allowed to react with formaldehyde (HCHO), which gradually developed a yellow color, owing to the synthesis of diacetyldihydrolutidine. The intensity of the yellow colored complex was measured at 412 nm in a spectrophotometer. The AOX activity was estimated from a formaldehyde (μmol/mL) standard liner curve plotted earlier.

2.3. Assay for the alcohol oxidase enzyme by the acetylacetanilide method - the formaldehyde approach

Assay for the AOX activity was carried out in the 96-well microplates (UV-Star® Microplates, Greiner Bio-One, USA). To each well was added 62.5 μL of the culture filtrate of the fungi grown on the KL and LMCs, a 100 mM concentration of methanol as the substrate and the mixture was incubated at room temperature for 15 min. The amount of formaldehyde released as a sequel to the oxidation by the AOX of methanol was determined by allowing it to react with the acetylacetanilide reagent (25 μL 0.59 M acetylacetanilide reagent in DMSO/water, 80/20 *v*/v). The reaction mixture was incubated for 15 min at room temperature, and the intensity of the fluorescence of the compound formed was measured at 460 nm (excitation wavelength at 360 nm) in a Microtiter Plate Reader (BioTek, Synergy HT, USA). The AOX activity was, then determined from a formaldehyde (μmol/mL) standard liner curve, plotted earlier.

2.4. Validation of the acetylacetone and the acetylacetanilide methods

In order to validate the acetylacetone and the acetylacetanilide methods, in the presence of methanol by means of a commercial alcohol oxidase (AOX; EC 1.1.3.13 solution from *Pichia pastoris* -the buffered aqueous solution contained 10–40 units/mg protein (biuret), A2404 Sigma-Aldrich) that could oxidize methanol and generate formaldehyde was used. The formaldehyde that was produced by way of the methanol oxidation or the oxidation of formaldehyde forms complexes by making use of acetylacetone and acetylacetanilide provided. The Standard curves were plotted by employing the (a) acetylacetone and (b) acetylacetanilide at various concentrations. The determination of the AOX

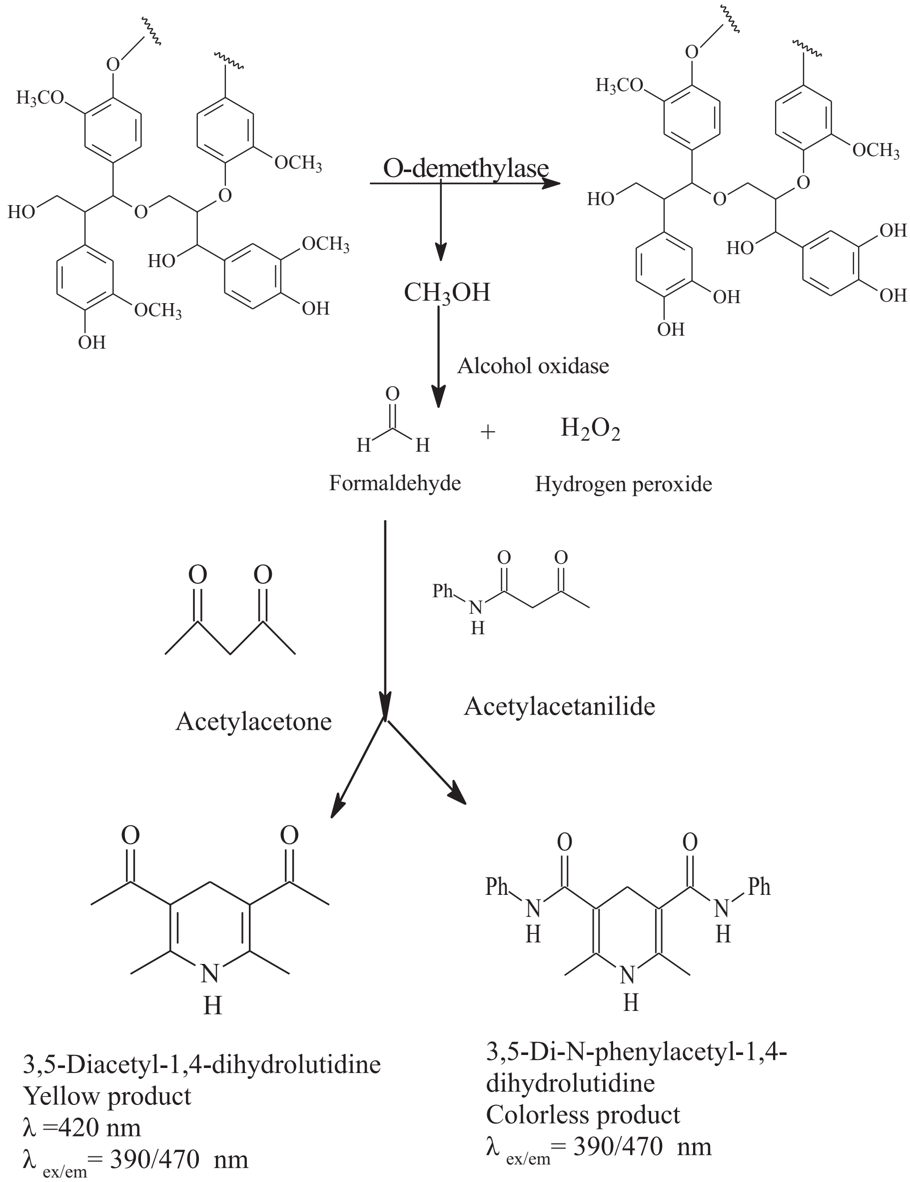

Fig. 1. Novel methods for detecting alcohol oxidase activity using acetylacetone and acetylacetanilide was developed using commercial alcohol oxidase from Sigma-Aldrich using methanol as substrate. In microbial lignin demethylation step which strips-off *O*-methyl (−CH_3) groups from an aryl methoxyl, (i.e., methoxyl substitution) and produces primarily methanol, with the participation of lignin specific *O*-demethylases or ligninolytic enzymes.

activity, by means of the methods devised by the use of acetylacetone and acetylacetanilide and by the use of the commercial alcohol oxidase to oxidize different primary alcohols such as methanol, ethanol, isopropanol, 2-methoxy ethanol, 1-propanol and n-butanol, as the variable alcoholic substrates, in order to validate the methods that were devised for the purpose.

3. Collection and isolation of the fungal taxa

Wood-rot fungi were collected and were isolated from the Boreal forests (Northern Ontario Boreal forest, Canada) These were purified adequately and were maintained on the PDA slants at 4 °C for further use. The Vogel Minimum Salts Media ('VMSM 50 × salts; Na_3 citrate, 5.5 H_2O – 150 g, KH_2PO_4, anhydrous – 250 g, NH_4NO_3, anhydrous – 100 g, $MgSO_4$, 7 H_2O – 10 g, $CaCl_2$, 2 H_2O – 5 g, 5 ml of trace elements solution (citric acid, 1 H_2O – 5 g, $ZnSO_4$, 7 H_2O– 5 g, $Fe(NH_4)_2$, 6 H_2O – 1 g, $CuSO_4$, 5 H_2O – 0.25 g, $MnSO_4$, 1 H_2O – 0.05 g, H_3BO_3 anhydrous – 0.05 g, Na_2MoO_4, 2 H_2O – 0.05 g), biotin solution – 2.5 mL, distilled water – 1 L) was diluted with the 1 × media containing a 0.2% of glucose solution and a 0.3% Kraft lignin (KL) was used as the substrate (pH 5.8). The media was sterilized at 121 °C for 20 min. The culture media was inoculated with the initial 450 fungal isolates rose aseptically earlier and were incubated at 28 °C for a month in order to evaluate their lignin degrading ability. The best lignin degrading cultures, numbering 101 were identified, based on their lignin decolorizing percentage values. Out of these, the potential ligninolytic fungi, numbering 16, which were identified on the basis of the degree of their ligninolytic pontial were chosen for screening them for their ability to secrete the alcohol oxidase enzyme during the course of the KL and LMC degradation process.

3.1. Evaluation of the alcohol oxidase enzyme secreting potential of the test fungi

The potential ligninolytic fungi (16) that were chosen earlier, were taken up for screening them for their ability to secrete the enzyme, AOX during lignin degradation as well as the LMCs during the lignin-demethylation process, as induced by the Kraft lignin (KL) and the veratryl alcohol as the carbon sources (Table 2). Two different type of media were prepared using i) a 0.3% KL as the carbon source and ii) a 30.4 mM veratryl alcohol in the VMSM (pH 5.8). The nutrient media were sterilized at 121 °C for 20 min. The lignin demethylating fungi were inoculated into the minimal Vogels Salt media and the methanol generated by the action of the KL and the LMCs served as the carbon source for further alcohol oxidase secretion. The AOX activity was determined by using methanol as the substrate and the formaldehyde that was generated was detected and measured by way of the a) acetylacetone and (b) acetylacetanilide methods. The AOX enzyme activity was estimated by the extent of methanol oxidation and next, the formaldehyde that was released was quantified as that activity of μmol / mL of the AOX enzyme (U/mL).

3.2. Assessment of the AOX enzyme secreting ability of Aspergillus sp.3 (BRI 270) by the acetylacetone method

In this investigation, we have identified and chosen a lone *Aspergillus* sp.3 (BRI 270) as a potential candidate for the lignin demethylation studies on the basis of its ability to demethylate lignin by upto 31%. The organism was allowed to grow on the following lignin based model compounds, such as 2,6-dimethoxyphenol, 3-hydroxyl-4-methoxy cinnamic acid, 4-hydroxy-3-methoxy cinnamaldehyde, syringaldehyde, *o*-vanillin, syringic acid, guaiacol, vanillin, ferulic acid, veratryl alcohol and the Kraft lignin (KL) along with some of the metabolic intermediates and carbon sources, such as glucose (fermentation sugar), H_2O_2, ethanol, isopropanol, n-butanol etc. Methanol was also checked for its AOX inducing ability if any, when incorporated into the minimal Vogels Salt media (pH 5.8). The media were sterilized at 121 °C for 20 min. *Aspergillus* sp.3 (BRI 270) was inoculated into the sterilized media, and was incubated at 28 °C, in a rotary shaker incubator revolving at a speed of 180 rpm for 7 days. The methanol that was generated served as the carbon source for the secretion of the alcohol oxidase enzyme and the AOX activity was determined with methanol also serving as a substrate. The crude culture filtrate contains the AOX enzyme was able to oxidize and generate certain formaldehyde complexes in the presence of methanol, by making use of acetylacetone. The activity of the AOX enzyme has been expressed as the U/mL.

4. Results and discussion

The valorization value of the biomass feedstock has been observed to have risen considerably by employing the wood-rot fungi, such as *Phanerochaete chrysosporium*, the brown-rot and the white-rot fungi, besides the bacteria, all of which are known to secrete a wide range of accessory enzymes, including the alcohol oxidase, glyoxal oxidase and the aryl-alcohol oxidase during the degradation of the biomass also generating hydrogen peroxide (H_2O_2). Further, the enzyme oxidizes and generates the free radicals that act on cellulose, hemicellulose and lignin, producing a modified polymer or alternatively it may degrade it into its constituent monomers (Bourbonnais and Paice, 1988; Bourbonnais and Paice, 1992; Chinnadayyala et al., 2015; Kuwahara et al., 1984; Marzullo et al., 1995; Okamoto and Yanase, 2002; Romero et al., 2007; Sannia et al., 1991). Of the various enzymes that are secreted during the course of the degradation process, the AOX enzyme alone appears to catalyze the oxidation process of alcohols significantly, thereby giving rise to an aldehyde and hydrogen peroxide. The hydrogen peroxide (H_2O_2) that is generated activates the peroxidases, which vigorously attack the lignin, subsequently paving the way for the demethylation, hydroxylation, and oxidation, besides a depolymerization event (Martinez et al., 2011). The AOX enzymes are produced in a few basidiomycetes, such as *Pleurotus sajor-caju, Pleurotus eryngii*, *Bjerkandera adusta*, *Gloeophyllum trabeum*, *P. chrysosporium*, and *Penicillium purpurescens* (Asada et al., 1995; Marzullo et al., 1995; Okamoto and Yanase, 2002; Sannia et al., 1991; Romero et al., 2007; Daniel et al., 2007; Isobe et al., 2009; Hernandez-Ortega et al., 2011). The AOX enzyme essentially catalyzes the oxidation of certain common alcohols like methanol, ethanol, 1-propanol, glycerol and the lignin-related aryl-alcohols (veratryl-alcohol) (Linke et al., 2014). Further, the AOX displays a substrate specificity towards methanol, which is secreted by *G. trabeum*, *P. pastoris*, and *P. variotii* which exhibited their Km values of 2.3, 1.4 and 1.9 mM, respectively (Daniel et al., 2007; Kondo et al., 2008; Couderc and Baratti, 1980). However, the current method to measure the AOX activity, based on AOX-POD-ABTS method, does not appear to be suitable for detecting the AOX in some biological samples containing the laccases (LAC) or peroxidases (POD). Because, these ligninolytic enzymes are believed also to catalyze a simultaneous oxidization of the ABTS (2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)) leading to considerable ambiguity of the facts, even to the extent of misleading the investigations into believing that the observations are a sequel to the activity of the AOX enzyme. Hence, we have developed two methods to detect the AOX activity, (i) neutral solutions of acetylacetone and the ammonium salt that react with formaldehyde (HCHO), gradually developing a yellow colored complex, owing to the synthesis of the diacetyldihydrolutidine, whose intensity was estimated by measuring the absorbance of the product at 412 nm (Fig. 1). In the second method, the amount of formaldehyde formed because of AOX facilitated oxidation of methanol was determined by reacting it with the acetylacetanilide reagent, and the intensity of the fluorescence of the product formed was measured at 460 nm (excitation wavelength at 360 nm) in a spectrofluorometer. The related methods for the qualitative and quantitative determination of the alcohol oxidase enzyme are described elsewhere.

The oxidation process of methanol by the AOX enzyme has been observed to produce an equivalent of hydrogen peroxide (H_2O_2) on addition of the horseradish peroxidase and an appropriate chromogenic peroxidase substrate, such as 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). The amount of H_2O_2 generated by the AOX reaction could be quantified. Several investigators have adopted this AOX/peroxidase/ABTS system as a reliable means to quantify methanol (Herzberg and Rogerson, 1985; Mangos and Haas, 1996). However, those methods that are based on the hydrogen peroxide (H_2O_2) quantification are not adequate enough as specific methods for the detection of the methanol content or for estimating the AOX activity, because the hydrogen peroxide is known to be produced even by the other ligninolytic enzyme actions as well. Besides, the available colorimetric and fluorimetric methods for quantifying the aldehyde product with acetylacetone or the fluoral-P are relatively more specific for formaldehyde than for the other aldehydes (Nash, 1953; Compton and Purdy, 1980). Although, the combination of the AOX and a specific formaldehyde reagent provides an appropriate one for the specific oxidation of methanol, formaldehyde also is observed to serve as an apt substrate for the active functioning of the AOX enzyme. Further, it gets oxidized to formic acid even as an additional equivalent of hydrogen peroxide is produced from formaldehyde (Hopkins and Muller, 1987; Patel et al., 1981). Interestingly, in a similar method, Klavons and Bennett (1986) appear to have assayed the pectin methylesterase (PME) by the AOX enzyme and acetylacetone to quantity the methanol that is generated by a microbial degradation process (Blumer et al., 2000; Van den Broeck et al., 2000). In yet another method, the MBTH has been used in conjunction with the AOX for the determination of methanol and it has been considered to be a relatively simple and sensitive method in order to evaluate the PME activity. As per a recent study on the lignin, the demethylation process appears to have prompted an overexpression of the AOX enzyme in several brown-rot (BR), white-rot, and the soft-rot fungi that mediate the demethylation events of lignin and the LMCs, thereby producing a lignin which has been more phenolic in nature (Eriksson et al., 1990; Jin et al., 1990; Martinez et al., 2011; Filley et al., 2002). However, the brown-rot and white-rot fungi have been observed to secrete a higher degree of the AOX enzyme that

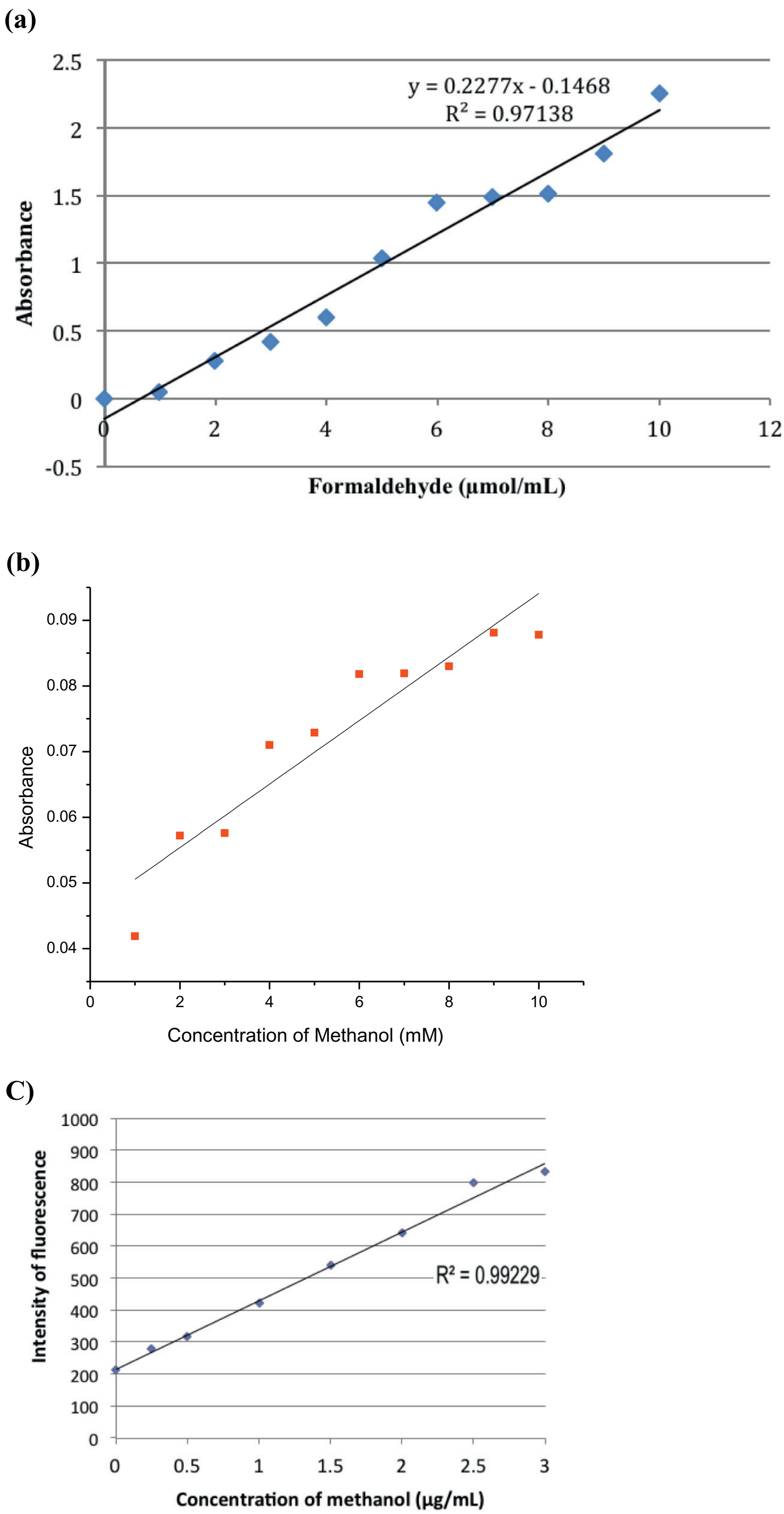

Fig. 2. The Standard curves developed using (a) Formaldehyde detection using acetylacetone and (b) Formaldehyde generated from methanol by AOX enzyme detection using acetylacetone c) methanol oxidized generated formaldehyde detection using acetylacetanilide methods using commercial alcohol oxidase from Sigma-Aldrich, Canada.

oxidizes the methanol, thereby producing formaldehyde (HCHO) and hydrogen peroxide (H_2O_2), which play a vital role in the lignin decay process by these organisms (Filley et al., 2002; Daniel et al., 2007; Martinez et al., 2009; Martinez et al., 2011).

In order to determine the AOX activity in the biological materials, a standard curve with various concentrations of formaldehyde and methanol (1 to 10 μmol/mL) was plotted. The methanol was allowed to be oxidized by means of a commercial AOX enzyme, procured from the Sigma-Aldrich and the formaldehyde that was released was quantified, so as to plot a standard graph. At the same time, various concentrations of formaldehyde (HCHO) were employed in order to plot the standard curves. Two methods were used: In the first method, the neutral solutions of acetylacetone and the ammonium salt were allowed to react with formaldehyde (HCHO), which leads to a gradual development of a yellow color, owing to the synthesis of the diacetyldihydrolutidine and the product, thus generated was measured at 412 nm in a spectrophotometer (Fig. 2.). In the second method, the amount of formaldehyde generated as a consequence of the AOX catalyzed oxidation of methanol was determined by allowing it to react with the acetylacetanilide reagent, and the fluorescence emitted by the product was estimated by measuring its intensity at 460 nm (excitation wavelength at 360 nm) in a spectrofluorometer (Fig. 2).

In order to evaluate the efficacy of the acetylacetone and acetylacetanilide methods, the commercial AOX enzyme (Sigma) was used as the catalyst to oxidize some primary alcohols serving as the substrates, such as the isopropanol, 2-methoxyl ethanol, 1-propanol, ethanol, n-butanol, and methanol and the product of oxidation was measured by way of the spectrophotometric methods involving both acetylacetone and acetylacetanilide. Of the various substrates tested, the AOX enzyme essentially oxidized methanol and generated formaldehyde, and further reacted with acetylacetone to produce a yellow colored complex that gave rise to 1402 U/mL of the enzyme (Table 1). But the other primary alcohols tested by the acetylacetanilide method did not show appreciable levels of activity (Table 1). The efficacy of this novel acetylacetone assay for the determination of the AOX enzyme activity during the lignin demethylation process was investigated further. For this purpose, the potential (16) lignin demethylating fungi were screened for their ability to release methanol during the demethylation of lignin or the LMC demethylation process and those that induced the alcohol oxidase production were evaluated further for their efficacy. Hence, we tested the lignin-demethylation efficacy and the AOX secretion ability of the test organism as induced by the Kraft lignin (KL) and veratryl alcohol serving as the carbon source. The AOX activity was determined by incorporating acetylacetone and by measuring the formaldehyde that was generated by an oxidative process of methanol with the KL and the veratryl alcohol serving as the substrates. The results are expressed as μmol / mL of formaldehyde generated. The maximum alcohol oxidase activity was observed with *Galerina autumnalis* LU86 (29 U/mL), *Ctenomyces serratus* LU122 (83 U/mL), *Aspergillus* sp.1 BRI122 (48 U/mL), *Penicillium* sp. 2 BRI269 (23 U/mL), *Aspergillus* sp. 3 BRI270 (30 U/mL) and *Botryosphaeria rhodina* BRI274 (42 U/mL) that were reared in the presence of the Kraft lignin (KL) (Table 2). Whereas, the brown-rot genome sequences have revealed that the alcohol oxidase genes are overexpressed and that they play a crucial role in the wood degradation process (Martinez et al., 2009, 2011**).** In a recent study with an *Ochrobactrum* sp. AIU 033, it has been observed that a higher concentration of glyoxylate from the glycolate accumulates, but the enzyme does not seem to oxidize the glyoxylate, ethylene glycol, glycerol, or methanol (Yamada et al., 2014). In the case of the veratryl alcohol raised cultures, none of them seems to display significant levels of the AOX enzyme activity except *Botryosphaeria rhodina* BRI274 (32 U/m), while a few test fungi grown in the presence of veratryl alcohol have displayed the least alcohol oxidase activity. These are the organisms studied - *Gliocladium roseum* LU08 (8 U/mL), *Gliocladium roseum* LU25 (12 U/mL), *Sporobolomyces roseus* LU29 (16 U/mL), *Cylindrocladium camelliae* LU120 (12 U/mL) and *Ascodesmis microscopia* LU131 (18 U/mL) (Table 2). Recently, an AOX mediated gold nanoparticles were successfully synthesized and stabilized (Reddy et al., 2015).

Table 1
Novel method development using commercial alcohol oxidase from Sigma-Aldrich, Canada to oxidize different primary alcohols using acetylacetone and acetylacetanilide methods.

Primary alcohols	Alcohol oxidase activity (U/mL)	
	Acetylacetone[a]	Acetylacetanilide[b]
Methanol	1402 ± 3.51	137.67 ± 9.84
Ethanol	81.33 ± 2.85	8 ± 0.58
Iso-propanol	43 ± 1.16	9.67 ± 1.20
2-methoxyl ethanol	53. 7 ± 1.45	7.67 ± 0.33
1-propanol	56 ± 1.53	7.67 ± 0.33
n-butanol	131 ± 1.14	7 ± 0.43

[a] The AOX reaction started by adding 100 μl of enzyme and incubated with methanol (replaced with primary alcohols), mix gently and vortex incubated at 25 °C for 15 min, followed by 100 μl of acetylacetone, mix well and incubated at 60 °C for 15 min. The yellow color formation was measured at 412 nm.

[b] The amount of formaldehyde formed because of AOX oxidation of methanol was determined by reacting with acetylacetanilide reagent (25 μL 0.59 M acetylacetanilide reagent in DMSO/water, 80/20 *v*/*v*). The reaction was incubated for 15 min at room temperature, and the fluorescence of the product formed was measured at 460 nm (excitation wavelength at 360 nm) in a Microtiter Plate Reader.

Table 2
Alcohol oxidase activity determination by acetylacetone method using Kraft lignin and veratryl alcohol as substrates.

Lignin demethylating fungi	Alcohol oxidase activity (U/mL)[a]	
	Veratryl alcohol	Kraft lignin
Ascodesmis microscopia (LU131)	18 ± 0.03	25 ± 0.05
Aspergillus sp.1 (BRI 122)	ND	48 ± 0.03
Aspergillus sp.3 (BRI270)	24 ± 0.01	30 ± 0.02
Botryosphaeria rhodina (BRI274)	32 ± 0.06	42 ± 0.07
Ctenomyces serratus (LU122)	ND	83 ± 0.05
Cylindrocladium camelliae (LU120)	12 ± 0.02	60 ± 0.01
Entoloma sp. (LU89)	ND	38 ± 0.1
Epicoccum purpurascens (LU33)	ND	24 ± 0.04
Galerina autumnalis (LU86)	ND	29 ± 0.2
Gliocladium catenulatum (LU111)	ND	27 ± 0.1
Gliocladium roseum (LU08)	8 ± 0.06	18 ± 0.03
Gliocladium roseum (LU25)	12 ± 0.01	30 ± 0.02
Gliocladium viride (LU124)	ND	82 ± 0.01
Penicillium thomii (LU32)	ND	25 ± 0.1
Penicillium sp.2 (BRI269)	ND	23 ± 0.4
Sporobolomyces roseus (LU29)	16 ± 0.08	15 ± 0.1

[a] The two different type of media were prepared using i) 0.3% KL as carbon source and ii) 30.4 mM of veratryl alcohol in minimal Vogels salt media were prepared as substrate (pH 5.8). The media were sterilized at 121 °C for 20 min. The lignin demethylating fungi was inoculated into minimal Vogels salt KL and LMCs generated methanol was served as carbon source for alcohol oxidase production and AOX activity was determined using methanol as substrate in enzymatic reaction and generated formaldehyde were detected using a) acetylacetone and (b) acetylacetanilide methods.

Out of the several fungi tested, we chose *Aspergillus* sp.3 (BRI270) as a potential demethylating candidate to strip-off the $-OCH_3$ groups in the lignin molecule that could produce methanol, and induce the secretion of the AOX enzyme (Table 3). Along with various LMCs, the primary alcohols and the Kraft lignin (KL) were used to check for the AOX activity by using *Aspergillus* sp.3 (BRI270). This isolate displays the highest AOX elaboration in situ with guaiacol (1684 U/mL) followed by Vanillin (102 U/mL), glucose (109 U/mL), ethanol (120 U/mL) and methanol (93 U/mL) as the substrates (Table 3). Both the methods have been specific towards formaldehyde that was generated during the oxidation process of methanol, as catalyzed by the AOX enzymes. For the qualitative and quantitative determination of methanol or ethanol (blood alcohol), butanol or ethanol from the fermented materials in the radioiodine labeling procedures, or at the biorefinery and biodiesel industries, the acetylacetone and acetylacetanilide methods have been observed to be sufficiently efficacious and rapid, besides being sensitive

Table 3
AOX activity determination using the lignin demethylating fungus *Aspergillus* sp.3 (BRI270) by acetylacetone method.

Lignin-like compounds[a]	Alcohol oxidase activity (U/mL)
2,6-Dimethoxyphenol	104 ± 0.03
3-Hydroxyl-4-methoxy cinnamic acid	94 ± 0.02
4-Hydroxy-3-methoxy cinnamaldehyde	93 ± 0.05
Syringaldehyde	92 ± 0.14
O-vanillin	100 ± 0.01
Syringic acid	30 ± 0.015
Guaiacol	1684 ± 0.32
Vanillin	114 ± 0.09
Ferulic acid	27 ± 0.22
Veratryl alcohol	102 ± 0.10
Kraft Lignin	96 ± 0.03
Metabolic intermediates and other carbon sources[a]	
Glucose	109 ± 0.12
H_2O_2	87 ± 0.08
Ethanol	120 ± 0.32
iso-propanol	106 ± 0.03
n-butanol	84 ± 0.06
Methanol	93 ± 0.001

[a] The lignin-like compounds and metabolic intermediates was added as AOX inducers in minimal Vogels salt media were prepared as a substrate (pH 5.8). The media were sterilized at 121 °C for 20 min. The *Aspergillus* sp.3 (BRI270) used to detect AOX activity, inoculated in sterilized media, and incubated at 28 °C, 180 rpm for 7 days.

than those methods which rely upon the GC and HPLC analyses (Goswami et al., 2013).

Over the last few decades, a chemical approach has been in vogue to detect the synthesis of formaldehyde (HCHO) by incorporating the permanganate to oxidize methanol to yield the formaldehyde, which could be estimated by means of colorimetry (Table 4). Various other approaches have also been made in order to detect and quantify both formaldehyde and methanol from the biological as well as the environmental sources. These methods are as follows: (i) formaldehyde based detection method (Sritharathikhun et al., 2005), acetoacetanilide method (Li et al., 2007a; Li et al., 2007b; Li et al., 2008), 4-Amino-3-penten-2-one (Fluoral P) method (Tsuchiya et al., 1994), 5,5-Dimethycyclohexane-1,3-dione method (Sritharathikhun et al., 2005), Spectrofluorimetry method (Gholami et al., 2016) etc. These apart, the acetylacetone (pentane-2,4-dione) method, the Fluoral-P (4-amino-3-penten-2-one), the Purpald (4- amino-3-hydrazino-5-mercapto-1,2,4-triazole) method, the *N*-methylbenzothiazolinone-2-hydrazone (MBTH) method, and the Fe^{3+} added (Klavons and Bennett, 1986; Nash, 1953; Nemecek-Marshall et al., 1995; Belman, 1963; Jacobsen and Dickinson, 1974; Avigard, 1983; Zurek and Karst, 1997; Hopps, 2000) method have also been put to effective use. Certain methanol detection methods, such the Abbe Refractometry (de G'oes et al., 2016), Raman spectroscopy (de G'oes et al., 2016), the Microfluidic Distillation System (Wang et al., 2016), the MoSe2 nanosheets (Nagarajan and Chandiramouli, 2018) have also been utilized by various investigators. Interestingly, in the CO_2 laser scribe, a Polymethyl-Methacrylate Microfluidic Chip Reaction occurs within the three-dimensional circular chamber, where the contents are mixed in a vortex device with methanol and methanol oxidase (MOX) and as the heat generates, the formaldehyde that is generated, reacts with the fuchsin-sulphurous acid, after which the microchip is loaded on to a UV spectrophotometer for the purpose of methanol detection (Wang et al., 2012). Besides, an other system, that is again mediated by the alcohol oxidase (AOX) enzyme generates hydrogen peroxide (H_2O_2), which then oxidizes a capping agent - cysteine (CSH), wihich in turn produces the CSH-stabilized cadmium sulphide quantum dots (CdS QDs) (Barroso et al., 2018). However, the determination of the AOX activity by the use of acetylacetone and acetylacetanilide could serve as a viable alternative to the conventional alcohol oxidase-peroxidase- 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid (AOX-POD-ABTS) based method. In view of this, this method appears to be invaluable for application at the various food, pharmaceutical, and biorefinery industries.

Table 4
Comparison of various methods and their potential in formaldehyde, methanol and alcohol oxidase activity determination.

Reagents or Instrument methods	Reaction temperature (°C)	Formaldehyde/methanol working range	References
Formaldehyde detection			
Acetylacetone	60	1.5–15 × 10^{-7} M	Sritharathikhun et al., 2005;
Acetoacetanilide	25	0.5–40 × 10^{-7} M	Li et al., 2007a; Li et al., 2007b; Li et al., 2008
4-Amino-3-penten-2-one (Fluoral P)	60	0.5–100 × 10^{-6} M	Tsuchiya et al., 1994
1,3-Cylohexanedione	95	0.14–1.4 × 10^{-7} M	Fan and Dasgupta, 1994
5,5-Dimethycyclohexane-1,3-dione	130	1.7–3.3 × 10^{-7}, and 0.83–3.3 × 10^{-7} M	Sritharathikhun et al., 2005
Spectrofluorimetry	*25 °C, 8 min	–	Gholami et al., 2016
Methanol detection			
Abbe refractometer	Room temperature	10 mL, accuracy depends on this volume (picnometer volume)	de G'oes et al., 2016
Raman spectroscopy	16–27 °C	Depends on the optics, for micro cuvettes less than 1 mL	de G'oes et al., 2016
Microfluidic distillation system using a spectrophotometer	–	300–800 ppm	Wang et al., 2016
MoSe2 nanosheets	–	–	Nagarajan and Chandiramouli, 2018
Fluorescence spectroscopy/photoelectrochemical (PEC) analysis	Room temperature	two alcoholic solutions with ethanol content of 40% and 6% of methanol	Barroso et al., 2018
Alcohol oxidase activity			
Acetylacetone/Acetylacetanilide[a]	25 ± 1 °C	10–100 µmol/mL	Present study

[a] Kraft lignin demethylated by *O*-demethylases or ligninolytic enzymes, that generates the methanol serves as carbon source and induces AOX enzymes that produces formaldehyde. Further HCHO reacts with (i) neutral solutions of acetylacetone and ammonium salt were react with HCHO, gradually develops a yellow color, owing to the synthesis of 3,5-diacetyl-1,4-dihydrolutidine (Yellow product; λ = 420 nm; $\lambda_{ex/em}$ = 390/470 nm) of the product generated were measured at 412 nm. Also (ii) in second method the amount of formaldehyde formed as a consequence of AOX oxidation of methanol was determined by reacting with acetylacetanilide reagent, and the fluorescence product of 3,5-di-*N*-phenylacetyl-1,4-dihydrolutidine (colorless product; $\lambda_{ex/em}$ = 390/470 nm) was measured at 460 nm (excitation wavelength at 360 nm).

5. Conclusion

In this investigation, an efficacious method has been devised for detecting the activity of the AOX enzyme from the various lignin demethylated and microbially fermentable materials that acts at a rapid pace. The AOX- oxidized methanol generates formaldehyde (HCHO) that reacts with acetylacetone and the ammonium salt, and a yellow color gradually develops, owing to the synthesis of the diacetyldihydrolutidine as the product, whose intensity was estimated by measuring its absorbance at 412 nm in a spectrophotometer. In an other method, the quantity of formaldehyde that accumulates as an oxidation product of the AOX- catalyzed oxidation process of methanol was estimated by way of reacting it with the acetylacetanilide reagent, and the intensity of the fluorescence of the product formed was measured by its absorbance at 460 nm (excitation wavelength at 360 nm) by means of a spectrofluorometer. Based on these observations, it is envisaged that the determination of the AOX activity by the acetylacetone and acetylacetanilide method, could perhaps replace the conventional AOX-POD-ABTS method. In view of this, it is proposed that this novel, sustainable method provides the much-needed alternative for applications at the various industries, including the food, pharmaceutical, fuel, biosensor, biorefinery, biopolymer, biomaterial, platform chemical, and biodiesel industries.

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