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# Novel application of coconut husk as growth support matrix and natural inducer of fungal laccase production in a bubble column reactor

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**Abstract.** Laccase production by a white-rot fungus *Pycnoporus sanguineus* and its growth in a bubble column reactor were studied as a function of different inducers and superficial gas velocities. Free suspended biomass and immobilized biomass systems were studied where in the latter novel application of coconut husk as simultaneous support matrix and natural laccase inducer was studied. Good biomass growth was observed in the free suspension cultivation but no laccase activity was detected even after known enzyme inducers were applied. In the immobilized biomass system supplied with aeration at superficial gas velocity of 1.14 x $10^{-3}$ m $s^{-1}$, the fungus growing on the surface of coconut husk produced significant amount of biomass at 2.4 ± 0.2 g $L^{-1}$ dry weight as compared to the non-aerated (control) system where only 0.6 ± 0.1 g $L^{-1}$ dry weight of biomass was obtained. Laccase activity was detected only after 36 hours of fermentation and the highest activity i.e. 2.85 ± 0.05 U $L^{-1}$ was obtained at 48 hours. No laccase activity was detected in the non-aerated system even after 48 hours of fermentation.

**Keywords:** Bubble column reactor, coconut husk, inducer, laccase, solid support, *Pycnoporus sanguineus.*

## INTRODUCTION

Laccase is a widely distributed enzyme found in many living organisms and its function is diverse among each of the organisms. This glycosylated enzyme (ϱ-diphenol dioxygen oxireductase, EC 1.10.3.2) belongs to the family of blue multicopper oxidases which reduce molecular oxygen to water and simultaneously perform one electron oxidation of various aromatic substrates such as diphenols, methoxy-substituted monophenols and aromatic amines (Thurston, 1994). Due to its relatively wide substrate specificity, laccase can participates and be a substitute to processes that mostly carried out either by chemical route with concomitant generation of toxic by-products or a physical process with high energy consumption. For example, laccase is currently used in paper pulp delignification (Camarero *et. al.*, 2007), treatment of wastewater containing phenolic compounds (Kurniawati and Nicell, 2008) and dye decolorization (Pricelius *et. al.*, 2007).

Biotechnological production of laccase enzyme is currently the subject of intense research. The effect of medium composition such as C:N ratio (Pointing *et. al.*, 2000), nitrogen level (Fu *et. al.*, 1997) and inducers (Gnanamani *et. al.*, 2006) have been studied. In fermentation process, different process conditions and reactor design such as stirred tank reactor (Fortina *et al.*, 1996), airlift reactor (Fenice *et al.*, 2003; Rancaňo *et al.*, 2003) and bubble column reactor (D'Annibale *et al.*, 2006; Quaratino *et al.*, 2007) were employed in order to improve the enzyme production.

In this short communication, we reported the novel application of coconut husk as a support matrix which acts simultaneously as a natural and environmental friendly inducer in the laccase fermentation using a bubble column reactor and the effect of superficial gas velocity on the process. Bubble column reactor was chosen because of its simple geometry as a gas–liquid contacting device which allows ease of operation and ability to provide good gas-liquid mixing and high heat transfer rate in a controlled manner (Jin *et al.*, 2007). A white-rot fungus, *Pycnoporus sanguineus* was used as laccase producer in this study. This strain was reported to produce laccase as the sole phenoloxidase in submerged culture (Pointing and Vrijmoed 2000).

## MATERIALS AND METHODS

*Fungal strain and culture maintenance.* *Pycnoporus sanguineus* Linn. Ex Fr (Murrill) was maintained and sub-cultured on potato dextrose agar (PDA) plates at 28±1°C for 7 days.

*Bubble column reactor and culture condition.* Basal medium for laccase production in bubble column reactor consisted of (per litre): 20.0 g of glucose, 2.0 g of

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yeast extract, 2.0 g of malt extract, 2.0 g of peptone, 1.0 g of $K_2HPO_4$, 0.5 g of $MgSO_4$, and 0.46 g of $KH_2PO_4$. The working volume for the bubble column reactor fermentation was 100 ml. For cultivation using biomass in free suspension, ten (10) fungal discs (10 mm diameter) from a fully grown *P. sanguineus* on PDA plates were used as inocula. For immobilized biomass system, 0.2 ± 0.02 g coconut husk cut into rectangular shape (150 mm (h) x 7 mm (l) x 7 mm (w)) was used and rendered static inside the bubble column reactor (immersed in the working volume) using a pivoted line. A glass column with 27 mm inner diameter and 410 mm height was used as the reactor. Experimental setup of the column reactor is shown in Figure 1. The effect of different superficial gas velocities on growth and enzyme production was studied at $1.14 \times 10^{-3}$, $2.29 \times 10^{-2}$ and $3.43 \times 10^{-2}$ m $s^{-1}$. The superficial gas velocity was calculated using equation (1):

$$\text{Supercial gas velocity (ms}^{-1}\text{)} = \frac{\text{volumetric gas flow rate (m}^3\text{s}^{-1}\text{)}}{\text{cross-sectional area of column (m}^2\text{)}}$$

(Eq. 1)

Volumetric oxygen mass transfer coefficient, kLa ($s^{-1}$) for different superficial gas velocities was calculated using static gassing-out method where the dynamic change in volumetric oxygen concentration with time was represented by equation (2):

$$\frac{dC_L}{dt} = k_{L\alpha} (C^* - C_L)$$

(Eq. 2)

where CL = bulk concentration of oxygen in liquid-phase; C* = liquid-phase concentration of oxygen in equilibrium with its concentration in gas-phase; t = time (s). The dissolved oxygen concentration was measured as its partial pressure in the liquid. Partial pressure measurement was made using Mettler-Toledo 12/200 A type oxygen electrode.

Upon integration of (Eq. 2),

$$\ln\left(1 - \frac{C_L}{C^*}\right) = -kL\alpha.\Delta t$$

(Eq. 3)

The $k_L a$ value was determined directly from the slope of $\ln\left(1 - \frac{C_L}{C^*}\right)$ against time plot.

Biomass weight and growth rate calculation. In free suspension system, dry biomass increase was determined at the end of the fermentation using gravimetric method. For immobilized system, it is impossible to separate the fungal biomass from the solid substratum. Thus, for fermentation runs using coconut husk as support matrix and inducer, the husk was taken out from the column, washed with distilled water and dried at 65°C to a constant weight. Then, biomass growth was determined from the difference between final dry weight and initial dry weight of the coconut husk. Specific growth rate (µ) was determined using equation (4):

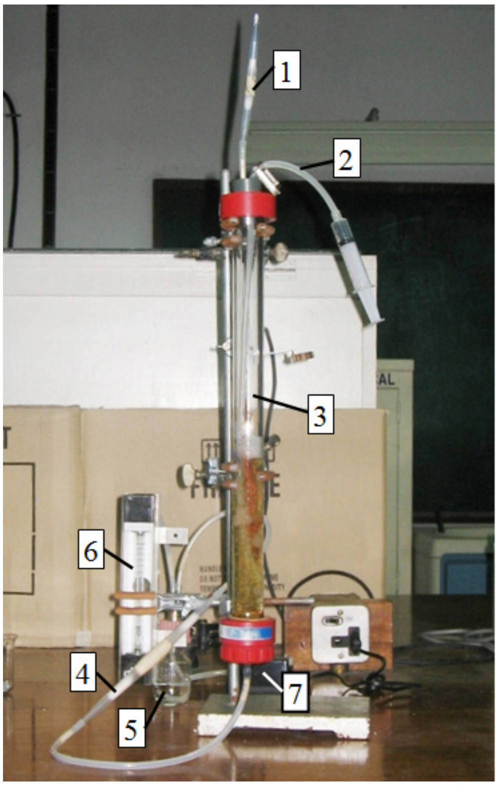

**Figure 1**. Photograph of bubble column reactor; 1-exhaust line, 2-sampling line, 3-column, 4-filter, 5-humidifier, 6- gas flow meter, 7- air pump

$$\mu = \frac{\ln X_{t2} - \ln X_{t1}}{t_2 - t_1}$$

(Eq. 4)

where, X is biomass weight (g); t is time (hour).

Average terminal settling velocity, $\upsilon g$ (m $s^{-1}$) for fungal plugs in the medium was calculated using equation (5):

$$\upsilon_g = \frac{d^2 (\rho_s - \rho) g}{18 \mu}$$

(Eq. 5)

where d : plug diameter (m); ϱs : plug density (g $m^{-3}$); ϱ : density of culture medium (g $cm^{-3}$); g : gravitational acceleration (9.81 m $s^{-2}$) and µ : viscosity of culture medium (g $m^{-1}$ $s^{-1}$). A minimum of 20 fungal plugs were measured to obtain their average diameter which was used in the calculation.

***Laccase assay.*** Laccase activity was determined using spectrophotometer at 525 nm with 0.1 mM syringaldazine in 50 mM sodium citrate buffer (pH 4.8) as substrate. Laccase activity was calculated as shown in equation (6):

$$\text{Laccase activity (UL-1)} = \frac{\Delta\text{Abs}}{t} \times \frac{1}{\varepsilon} \times \frac{1}{l} \times \frac{\text{total assay volume}}{\text{enzyme volume}}$$

(Eq. 6)

where, ΔAbs is the change of absorbance; t is the assay time (minutes); ε is extinction coefficient of syringaldazine

(65,000 $M^{-1}$ $cm^{-1}$) and l is the cuvette light path length (1 cm). One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μmol of syringaldazine per minute. The enzyme activity was expressed in enzyme unit (U) per litre (U $L^{-1}$).

***Statistical analysis.*** One-way Analysis of Variance (ANOVA) was performed on the experimental data obtained. A minimum of three (3) replicates was carried out for every measurement.

## RESULTS AND DISCUSSION

***Fungal growth in freely suspended biomass system.*** Different growth pattern was observed between the two cultivation systems viz. free suspended fungal biomass and immobilized fungal biomass on coconut husk. In the free suspension system, most of the fungal discs were suspended in the culture medium. Higher superficial gas velocity caused the discs movement to be more erratic due to intense bubbling than at lower velocities where a more regular upwards and downwards plug movement was observed. In the absence of aeration i.e. when the air-flow was switched off, average terminal settling velocity (calculated using Eq. 5) for a fungal disc was approximately 0.84 ± 0.04 m $s^{-1}$ ($n = 20$) and increased significantly as the fungal growth started to cover the plug's surface. After 24 hours cultivation, the plugs surfaces were fully covered by mycelia growth. Due to their increased mass, most of the fungal plugs were circulated near the bottom of the column after 36 hours of fermentation. Observable amount of freely suspended mycelia were present in the bulk liquid medium, probably sheared off from the static growth on the plugs by the liquid hydrodynamics. Most of the mycelia growth however was obviously confined to the plugs. Eventually at the end of 48 hours fermentation, the fungal plugs formed a single huge mass which impeded the rising of the air bubbles from the column sparger.

It is suggested that as the mass of the each single plug became heavier, their movement was restricted within a close proximity thus encouraged extensive mycelia linking across different plugs which finally formed a single fungal mass comprising of separate individual plugs. As a result, it was very difficult to obtain a reasonable estimate of the increase in fungal biomass weight at the end of the cultivations. Furthermore, in the free suspension system, laccase activity assay revealed a relatively low activity at < 0.1 U $L^{-1}$.

***Fungal growth in immobilized biomass system.*** Following the earlier observation, it is suggested that the free suspension system may not be a suitable system for *P. sanguineus* growth and laccase production in a bubble column reactor. It is hypothesized that an immobilized biomass system may results in an improved growth characteristics and favor laccase enzyme production by the fungus due to the fungus being able to grow in a more natural form. Immobilized system in this respect is defined as the provision of a solid matrix for supporting the mycelia growth of the fungus. Since simultaneous growth and laccase production by the fungus is desired, it is advantageous if the supporting matrix could also act as a natural inducer for the expression of laccase enzyme.

Coconut husk is one of the potential candidates that can served simultaneously as a support matrix for fungal growth; and a natural inducer for fungal ligninolytic enzymes production such as laccase due to its lignin content. It has multi-layer fibrous structure which offers high surface area without increasing the mass of the support. Due to the flexibility in its shape and structure, it can be easily incorporated into the bubble column. The coconut husk can also be used in its native form without requiring any pre-treatment step. Unlike in a stirred tank reactor (STR), the absence of mechanical movement in the bubble column eliminates the complication of incorporating such a solid support.

When *P. sanguineus* was cultivated using the immobilized system with coconut husk as support matrix, no freely suspended mycelia were observed in the liquid medium at all superficial gas velocities tested and in the absence of aeration; and subsequently no formation of a single huge fungal mass was observed in the column like the free suspension system. The application of different superficial gas velocities in the column did not yield significant differences in the final fungal biomass weight obtained i.e. ~ 2.40 g $L^{-1}$ ($p = 0.05$) (Table 1). The average specific growth rate (μ) calculated at different gas velocities tested was 0.114 ± 0.023 $h^{-1}$. However, in the absence of aeration (control), significantly lower biomass weight was obtained at 0.60 ± 0.06 g $L^{-1}$ (Table 1).

**Table 1.** Biomass weight of *P. sanguineus* after fermentation for 48 hours

| **Superficial gas velocity ($ms^{-1}$)** | **Biomass weight (g $L^{-1}$)** |
|---|---|
| $1.14 \times 10^{-3}$ | 2.40 x ± 0.21[a] |
| $2.29 \times 10^{-2}$ | 2.27 x ± 0.18[a] |
| $4.36 \times 10^{-2}$ | 2.36 x ± 0.50[a] |
| Control (no aeration) | 0.60 ± 0.60 |

a Results were not significantly different at $p = 0.05$

***Laccase production in bubble column reactor.*** In promoting the expression of laccase enzyme by *P. sanguineus* in bubble column fermentation, various operating conditions and known inducers were implemented (Table 2). However, none of the attempts yield satisfactory laccase activity as the enzyme assay revealed activity of < 0.1 U $L^{-1}$. For example, copper sulphate at the concentration of 0.01 g $L^{-1}$ and ethanol at 15.6 g $L^{-1}$, was used as proposed by Lomascolo *et al.* (2003) to boost the production of laccase in *Pycnoporus cinnabarinus*. However, in this study, no significant enhancement by these inducers was observed in the laccase production by *P. sanguineus*. This result suggests that the effect of inducer(s) to the production of laccase is strain specific and dependent.

Ryan *et al.* (2007) reported that nutrient limitation helps to induce significant laccase production in Trametes versicolor. Since the medium used in this study is an enriched formulation, replacement of 70% of the medium volume with sterile distilled water during the fermentation was carried out to create nutrient limitation condition (Table 2). This also failed to elicit significant expression of laccase enzyme. In combination with 0.01 g $L^{-1}$ copper sulphate, sawdust was also utilized in an attempt to induce the production of laccase enzyme which also resulted in almost nil laccase activity (Table 2). The difficulty in using sawdust was mainly technical due to its flotation to the surface because of its narrow density difference relative to the medium. This severely limited the accessibility of the sawdust to fungal biomass for effective induction. The problem was exacerbated when aeration was supplied as the floating sawdust gets deposited on the column inner wall and above the liquid level. Attempts to increase inoculum level and higher ethanol concentration in order to enhance the level of laccase expression by *P. sanguineus* were also not successful (Table 2).

When the fungal biomass was grown on the coconut husk as solid support, besides exhibiting good growth (Table 1), laccase production by the fungus was also significantly improved as shown in Table 3. It is suggested that coconut husk as solid matrix support for fungal growth simultaneously induced and/or enhanced the production of laccase enzyme as compared to the free suspension system. At 36 hours of fermentation, significant laccase activity was detected at all superficial gas velocities tested, however no laccase activity was detected before 36 hours. The highest activity of laccase assayed was 2.85 ± 0.05 U $L^{-1}$ after 48 hours of fermentation at 1.14 x 10-3 m $s^{-1}$ superficial gas velocity (Table 3). Statistical analysis however, revealed no significant difference in the biomass growth and the level of laccase activity expressed at different superficial gas velocities tested ($p = 0.05$). This showed that within the kLa range studied, growth and laccase production by the fungus were independent of volumetric oxygen gas supplementation (Table 3). It is clear that although there were significant increases in the kLa values (Table 3), this was not followed concomitantly in the significant increase of fungal growth and specifically laccase production. Thus, it is suggested that improved laccase production in particular can be attributed to the presence of coconut husks as inducer. For the control experiment (without aeration), no laccase activity was detected even after 48 hours of fermentation.

It was reported that coconut husk contains lignin at 46.5 ± 1.7 % of its dry weight (Bilba *et al.*, 2007). The total phenolics content of the husk material was determined to be 13.0 mg $g^{-1}$ dry weights, of which 18.5 to 19% is 4-hydroxybenzoic acid (4-HBA) and 1.3 to 1.7% was ferulic acid (Dey *et al.*, 2003). Ferulic acid was reported to play a significant role in the enhancement of laccase production by *P. sanguineus* (Vanhulle *et al.*, 2007). Thus ferulic acid may be one of the responsible components in the coconut husk that induced and/or enhanced laccase production in *P. sanguineus* in this study. It is suggested that coconut husk not only served as a good support for the fungal growth but also induced significantly its laccase production.

Further studies are currently being undertaken to examine in close detail the nature of application of coconut husk in the process and its reproducibility.

## CONCLUSIONS

Coconut husk offers an environmental friendly and economical alternative laccase inducer and/or enhancer in the laccase production process by white rot fungi in a bubble column reactor. The results obtained in this work illustrated the potential of employing such system in the production of ligninolytic enzymes by white rot fungi. The coconut husk not only allows the fungus to grow in its natural mycelia vegetative state, it also promotes the secretion of the ligninolytic enzyme like laccase as it contains phenolic compounds.

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**Table 2.** Various operating conditions and inducers applied to promote laccase production by *P. sanguineus* in a bubble column reactor

| Condition no | Plug no | Inducers | Superficial gas velocity ($ms^{-1}$) | Notes | Laccase production ($UL^{-1}$) |
|---|---|---|---|---|---|
| 1 | 10 | - | $1.14 \times 10^{-3}$ | | < 0.1 |
| 2 | 5 | - | $1.14 \times 10^{-3}$ | | “ |
| 3 | 5 | 0.01 g $L^{-1}$ copper sulphate & saw dust | $1.14 \times 10^{-3}$ | Saw dust was deposited around the column inner wall above the liquid level. | “ |
| 4 | 10 | 0.01 g $L^{-1}$ copper sulphate & 15.6 g $L^{-1}$ ethanol | $1.14 \times 10^{-3}$ | Inducers were added at the beginning of fermentation. | “ |
| 5 | 15 | 27.3 g $L^{-1}$ ethanol | $2.29 \times 10^{-2}$ | Inducer was added at the beginning of fermentation. | “ |
| 6 | 10 | 0.01g $L^{-1}$ copper sulphate & 15.6 g $L^{-1}$ ethanol | $1.14 \times 10^{-3}$ | 70 ml media was discharge and replaced with 70 ml distilled water with the inducers after 24 hours. | “ |
| 7 | 10 | 0.01g $L^{-1}$ copper sulphate & 35 g $L^{-1}$ ethanol | $1.14 \times 10^{-3}$ | 70 ml media was discharge and replaced with 70 ml distilled water with the inducers after 24 hours. | “ |

**Table 3.** Laccase production by *P. sanguineus* grown on coconut husk as solid support and laccase inducer

| Superficial gas velocity ($m\ s^{-1}$) | Laccase activity ($UL^{-1}$) | | kLa ($s^{-1}$) |
|---|---|---|---|
| | **36 hours** | **48 hours** | |
| $1.14 \times 10^{-3}$ | $1.62 \pm 0.08^{a}$ | $2.85 \pm 0.05^{a}$ | $0.016^{b}$ |
| $2.29 \times 10^{-2}$ | $0.52 \pm 0.05^{a}$ | $1.86 \pm 0.03^{a}$ | $0.025^{b}$ |
| $4.36 \times 10^{-2}$ | $2.09 \pm 0.21^{a}$ | $2.45 \pm 0.12^{a}$ | $0.034^{b}$ |
| Control (no aeration) | 0 | 0 | 0 |

[a] Results were not significantly different at $p = 0.05$

[b] Results were significantly different at $p = 0.05$, maximum standard deviation for all $k_La$ determination were <2 %.

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