Assessment of biomimetically sequestered calcium carbonate for tyrosinase production from *Pleurotus florida* with reference to phenol degradation: innovative approach for complete bioremediation of atmospheric CO_2

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***Abstract*—** Manuscript describes process to sequester CO_2 biomimetically and overcome problem to utilize its byproduct in environmentally benign way to produce mushroom and degrade phenol by the tyrosinase extracted from its different growth stages. Wastewater containing phenolic compounds presents a serious discharge problem due to their poor biodegradability, high toxicity and long term ecological damage. This study deals with both the aforementioned problems. CO_2 sequestration for its conversion into carbonate was pursued using electrometric unit. *Paenibacillus dendritiformis* (2.87 U/mg protein) showed a significantly high CO_2 sequestration capacity of 27.3 mg of $CaCO_3$/mg of Carbonic Anhydrase in 12 second. Biomimetic calcium carbonate was optimized for *Pleurotus florida* cultivation and all its stages were assayed for tyrosinase activity. Mushroom size (weight) was larger with substrate supplemented with 2% $CaCO_3$ with the yield of 74.2 g/150gm at mature stage. We finally determined that tyrosinase (8.9 U/mg) from premature stage degraded 90% phenol. GC-MS was castoff to identify the product formed after degradation of phenol by tyrosinase as Catechol. Mushroom grown using precipitated $CaCO_3$ shows the complete utilization of CO_2 with maximum phenol degradation from tyrosinase extracted from premature stage. The study holds tremendous economic implication of CO_2 sequestered product utilization, with strong potential utility of tyrosinase for subsequent decontamination of phenol.

***Keywords*—** *sequestration, Pleurotus, tyrosinase, phenol degradation, catechol*

I. INTRODUCTION

Upsurge in atmospheric greenhouse gas concentrations is accountable for a change in climate, for which there is a need to mend natural processes that confiscate atmospheric CO_2 [1]. In this context, the 'excess' CO_2 needs to be removed to compensate for anthropogenic emissions and maintain atmospheric levels at 500 ppm is of the order of 8 Gt C y−1 by 2050 [2]. For that, microbial CA have been used in conversion of CO_2 to calcium carbonate [3].The carbonates produced are thermodynamically stable and environmentally benign, thus minimizing the concerns over the monitoring requirements and long-term fate of CO_2 [1]. Although proved feasible it has a drawback in terms of the product disposal. In presence of suitable sources of calcium ions CO_2 sequestration capacities are expected be 10^3 - 10^5 tonnes CO_2 /year/cycle, upto ~3 mt CO_2 per year [4]. Thus it would be exceedingly lucid to congregate the produced calcium carbonate with a value added product, where it is completely utilized without any risk of CO_2 leakage. Thus, the noble approach to use precipitated calcium carbonate for production of *Pleurotus floria* has been castoff in this research plan. Pleurotus display inimitable developmental processes throughout its life cycles and its Developmental stages are strictly delimited by cellular signaling mechanisms including protein phosphorylation [5]. Calcium plays a universal role as an intracellular signal (second messenger) in filamentous fungi. Calcium is able to transducer a varied assortment of chemical or physical signals, consequential in a variety of cellular responses. Low extracellular Ca^{2+}concentrations lead to increased branching and diminish tip elongation, while concentrations of exogenous Ca^{2+} inhibit branching and induce hyphal elongation [6]. Western blot with antibodies anti-phosphotyrosine indicates a 92% increase of the phosphorylated state of a 43-kDa band, suggesting that the phosphorylation of protein is a component of the cascade that leads to the increase of tyrosinase activity in presence of calcium [7]. Tyrosinase (EC 1.14.18.1), copper-containing enzyme, catalyzes sequential oxidation steps with various phenolic substrates [8]. It catalyses monophenolase activity and diphenolase activity [9]. Tyrosinase-catalyzed oxidation is an ecofriendly and sustainable method to treat phenol contaminated wastewater [10]. Wastewater containing phenolic compounds boons a solemn discharge problem due to their deprived biodegradability, elevated toxicity and long term ecological damage. Hence, removal of phenols from industrial aqueous effluents is an important practical problem [11]. A major attraction of tyrosinase-mediated dephenolization over the processes catalyzed by other enzymes is that it does not require stoichiometric quantities of other reagents (such hydrogen peroxide used by peroxidases), apart from molecular oxygen as an oxidant [12]. The proposed research plan

envisages the development of a holistic approach for carbon dioxide sequestration involving the exploitation of carbonic anhydrase for biomimetic production of calcium carbonate, its subsequent utilization for mushroom production and application of mushroom tyrosinase for carbon dioxide sequestration through novel pathway involving the decontamination of phenol. Work is such designed that it assists sustainable and complete carbon dioxide removal systems to be trailed and possibly then implemented with the development of alternative method of removal of phenolic pollutant from industrial waste streams.

II. MATERIALS AND METHODS

A. Materials

Artificial sea water (ASW) was purchased from Sigma–Aldrich, St Louis, MO, USA. L-DOPA, potassium phosphate, tyrosine was purchased from Himedia Mumbai, India. All other chemicals were used of analytical grade and procured from Himedia, Mumbai, India.

B. Source of Microorganism

Pseudomonas fragi (BGCC# 1077), *Paenibacillus dendritiformis* (BGCC# 1159), *Bacillus licheniformis* (BGCC# 1166) *Oceanobacillus* (BGCC# 1186) were obtained from Bacterial Germplasm Collection Center (BGCC), Bacteriology laboratory, Dept. of Biological Sciences, Rani Durgavati University, Jabalpur (M. P.), India. These cultures were originally isolated from calcium carbonate kilns, Satna, M.P. India, lonar lake Maharashtra India and Lametaghat, Jabalpur M.P. India respectively. Fresh mushrooms (*Agaricus bisporus*, *Pleurotus florida*) were purchased from Food park industrial area, M.P. (India) while milky mushroom was purchased from INA market, Delhi, (India). Starting culture of *Pleurotus* was purchased from Directorate of Mushroom Research (Solan). The strain was maintained through periodic transfer at 4 °C on potato dextrose agar (PDA) plates after cultivation of mycelial blocks (5 × 5 mm) at 30 °C for 7 d.

C. Preparation of crude enzyme extract

Crude enzyme extract was prepared according to method of Bhattacharya [13] for which Cells (A600nm=1.2) grown in peptone broth (pH 8.0) were harvested by centrifugation at 10,000 rpm for 10 min at 4 °C. Cell pellets were suspended in Lysis buffer, pH 7.6 to a final concentration of 1.0 g (wet weight) of cells ml−1 and placed on ice for 10min at 4 °C. Following incubation, the cell extract was obtained by sonication (five short bursts of 10 s at 23 kHz followed by 30 s of cooling) of cell suspension in lysis buffer. Cell lysates were centrifuged at 8,000 rpm for 30 min, and supernatant was used as crude enzyme extract to determine carbonic anhydrase activity.

D. Determination of carbonic anhydrase activity

The method of Wilbur and Anderson [14] (1948) was followed with certain modifications. CO2 of standard grade was introduced at 1 bar (100 kPa) in 500 ml of Milli Q grade pure water for 1 h at 4 ◦C. CO2-saturated water (3 mL) was immediately added to 2mL of Tris–HCl buffer (100mM; pH 8.3) containing 0.5 mL of sample (before the assay test samples were immediately transferred to 4◦C following the ageing experiments with different pH, temperature and metal ions). The reaction vessel was maintained at 4°C. The time required for the pH to drop from 8.0 to 7.0 (t) was measured. The time required for the pH change (8.0–7.0) was used as control (tc) when buffer was substituted for the test sample. The Wilbur–Anderson Units were calculated with equation tc−t/t. The specific activity is expressed in Units/mL/mg protein.Protein concentrations were determined by the method of Lowry [15] with Bovine Serum Albumin as standard. Specific activity of enzyme was expressed as unit mg-1 protein. All the experiments were performed in triplicates.

E. Sequestration of CO_2 into calcium carbonate

The CO_2 saturated solution (distilled water and Artificial Sea Water) were prepared at room temperature. CO_2-saturated solution (10 mL) was released from reservoir and mixed with 1mL of Tris buffer (pH 8.3) in another section connected through a valve. The pH adjusted CO_2 saturated solution was allowed to react individually with CA (100g from 1mgmL^{-1} stock) from *Pseudomonas fragi*, *Ocenobaccilus and Paenibacillus dendritiformis*, and BCA for 15 min. The bicarbonate solution was released into another vessel through a valve containing 10 ml of $CaCl_2$ solution (at a final concentration of 0.01 mol L^{-1}). Tris buffer pH 9.5 (2 ml, 1M) was immediately added to the above mixture. The precipitation reaction was carried out by incubating the reaction mixture at 35°C and 45°C, respectively for 5 min and analyzing the amount of calcium carbonate formed by drying the sample and weighing the amount of calcium carbonate deposited. The same experiment was carried out in absence of enzyme and the amount of calcium carbonate precipitated was determined. The results were expressed in terms of mg $CaCO_3$ formed following control correction [1].

F. Determining the efficiency of the sequestration process

The sequestration efficiency in each experiment was evaluated by determining the ionic concentration of calcium present before and after carbonate precipitation. The results were expressed in terms of percentage efficiency of the calcium ion utilized [1].

G. Quantitative Imagining

For primary screening of tyrosinase activity, mushrooms was cut into two longitudinal halves and immediately solution of 0.002M of L-DOPA in 0.1 M TrisHCl buffer (pH 7.0) containing 0.1M sodium floride (pH 7.0) was sprayed in an homogenous way, on the cross section surface of the mushrooms and the time for appearance of red color was recorded [16]. The activity of the enzyme was determined by Secondary Screening , following Duckworth and Colman [17] through the formation of dopachrome spectrophotometrically [18]. One unit of tyrosinase was defined as the amount of the enzyme catalyzing the oxidation of 1 µmol L-DOPA to dopachrome per minute.

H. Optimization and Experimental design

Substrate formulation and Spawn preparation for Optimization of biomimetic calcium carbonate for *Pleurotus florida* production was done. Mycelia culture of *Pleurotus florida* was grown with different concentration of artificial and Biomimetic calcium carbonate (both at 0.5%, 1%, 1.5%, 2%, 2.5% and 3 %) following the method of Abdullah [19] with certain modification with moisture content 60% and the pH 6.0. Radial growth of the fungus was determined over 29 days. Spawn was prepared in Polypropylene bags filled with substrate (Wheat, 200 g) with different concentration of artificial and Biomimetic calcium carbonate (both at 0.5%, 1%, 1.5%, 2%, 2.5% and 3 %) and inoculated with eight days old mycelia culture of *Pleurotus florida* and were incubated at 28°C for 3 weeks [20]. Different formulation of substrate were prepared by varying calcium carbonate concentration from 0.5%, 1%, 1.5%, 2%, 2.5% to 3 % to prepare 18 replicate bags which were inoculated with 3 weeks old *Pleurotus florida* spawn, and incubated at room temperature in the dark at 28°C (19. Abdullah et al. 2013).After the appearance of fruiting bodies, mushrooms were harvested, counted and weighed. At the end of the harvest period (49 days), the accumulated data were used to calculate the BE and yield [21]. Estimation of calcium content was done titrimetrically. Calcium content was calculated as described by Masamba and Kazombo- Mwale [22].

Statistical software R was used to analysis the experimental data through box plot analysis and interaction was studied. Using R software box plots were created. All data are the mean of triplicates unless it is stated otherwise and their ± SD were almost negligible.

I. Effluent collection and Determination of phenol concentration

For Water from sewage pipeline carrying Industrial effluent was collected from outlet at industrial area Richhai, Jabalpur, Madhya Pradesh, India. Waste water samples were collected in amber bottles. It was acidified with H_3PO_4 to a pH of 4. The samples were kept at 4˚C [23]. Phenolic compound concentrations were determined by a colorimetric assay [24] based on the absorbance at 510 nm caused by the reaction between the phenolic compound, 4- amino antipyrine (AAP) and potassium ferricyanide [25].

J. Dephenolization by mushroom tyrosinase and identification of enzymatic end product

Mushroom extract (2 mL) was added to phenol (15 mL) solution and the reaction mixture was agitated at 100 rpm. 1 mL of mixture was withdrawn for every 6 hours, centrifuged to remove the precipitate formed and then assayed for phenol by UV-VIS spectrophotometer (2375 Double Beam Spectrophotometer). The phenol removal efficiency was assessed by determining the conversion as given by Xu and Yang [12]. The residue was analyzed by gas chromatography.The peaks were recorded with gas chromatography with high resolution mass spectroscopy (Instrument Name- Thermoscientific Gas Chomatography, Software- X caliber and Version- Qual browser 260514 E-33-PEF) and a 10µ syringe was used to load 1µ sample in helium column and 50 mM acetonitrile-phosphate buffer pH 7.0 was used as the mobile phase. The flow rate was 1 mL/min. The peaks were detected at 280 nm [26].

III. RESULTS

A. Sequestration of CO_2 into $CaCO_3$ and Evaluation of Carbonated Material

Significant tyrosinase enzyme activity was observed in *Paenibacillus dendritiformis* (2.87 U/mg protein) as compared to *Oceanobacillus* (2.13 U/mg protein), *Bacillus licheniformis* (2.11 U/mg protein) and *Pseudomonas fragi* (1.98 U/mg protein). The time taken for the initiation of the carbonation reaction with enzyme was clocked around 12–39 s while for uncatalysed reaction visual precipitation initiated after 63 s. In presence of CO_2-saturated distilled water, the amount of $CaCO_3$ formed (27.3 mg) and CO2 utilized (12mg) at 45 ◦C was highest in *Paenibacillus dendritiformis.*A positive correlation was observed for the amount of $CaCO_3$ formed and the amount of CO_2 utilized. CA isolated from different microorganisms is showing reasonably good CO_2 sequestration capacity compared to the CO_2 sequestration capacity of pure CA purchased from Sigma-Aldrich Co., St. Louis, MO.

B. Screening of mushroom for tyrosinase activity

Quantitative screening of mushroom revealed the non-homogenous distribution of mushroom tyrosinase. Part of stem connecting cap revealed presence of tyrosinase in slightly more

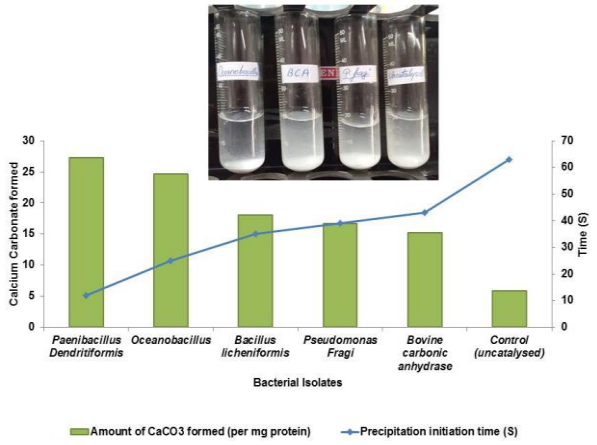

Fig. 1 Biomimetic sequestration of CO_2 into mineral carbonate **a)** Precipitation of CO_2 into $CaCO_3$ using indigenous *P. dendritiformis* CA, *Oceanobacillus* CA, *P. fragi* CA and commercial BCA under similar conditions. **b)** Summary of formation of carbonation material through biomimetic sequestration by microbial carbonic anhydrase

concentration. The distribution of tyrosinase activity seemed to be more homogenous in cap whereas other part showed even distribution of enzyme. All the four mushrooms were kept under similar condition and were screened qualitatively for tyrosinase activity (Fig-1). Quantity substantial tyrosinase activity was determined in two mushrooms they are Agaricus

bisporus and Pleurotus florida significant enzyme activity was observed in these isolates; Agaricus bisporus (6.112 Unit/mg), and Pleurotus florida (7.74 Unit/mg).

C. Evaluation of calcium carbonate for mushroom production

Selection of ideal concentration of calcium carbonate for substrate formulation of mushroom production was done by varying concentration of artificial and biomimetic calcium carbonate (both at 0.5%, 1%, 1.5%, 2%, 2.5% and 3 %).

Effect of substrate formulation

The colony color was white to beige and light yellow upon maturation. The formulation consisting of different percentage ratio of calcium carbonate among which 2% gave the highest mycelial growth rate followed by 2.5% and 3%. Wheat grains was found to be favouarable to mycelial extension and mycelial fresh weight of *Pleurotus florida*. The result shows that the mean fresh weight of mycelium grown using precipitated calcium carbonate was highest for 2% (1.318g) with 0.70 Unit/mg tyrosinase activity and 85.6mg/kg Calcium content. for artificial carbonate highest mycelium weight was 1.149g for 2% calcium carbonate with 0.68 Unit/mg tyrosinase and 81mg/kg Calcium content. The trend of day of incubation on different form of $CaCo_3$ on wheat of mycelial fresh weight for *P. florida* are the best combination.

Spawn Running

Noteworthy results were observed, With artificial calcium carbonate it took minimum 20 numbers of days for spawn running whereas minimum 15 days for spawn growth for precipitated calcium carbonate.

Emergence of Primordial

Calcium carbonate treatments showed results in terms of days taken for emergence of primordial after completion of mycelial growth. Primordial grown using precipitated calcium carbonate took minimum 15 numbers of days, whereas 19 days respectively to reach primordial initiation using artificial calcium carbonate. Among the various CaCO3 concentration 2% CaCO3 took minimum number of days to complete emergence of primordial but 2% precipitated calcium carbonate was recorded best in terms of time taken (in days) for emergence of primordial by taking minimum number of days.

Premature Stage

Pleurotus behaved significantly on different form and concentration of calcium carbonate to attain premature stage after young stage. Significant result was obtained with 2% calcium carbonate utilization. It took minimum 9 days with 8.3gm/Unit weight, 8.9 units/mg tyrosinase activities per 3 gram and 25.4(mg/100gm) calcium content. Among the various concentration of calcium carbonate used for growth of *P. florida* 2.5% and 3% .

Mature stage

Pleurotus florida grown on 2% precipitated calcium carbonate showed 7 fruit bodies with yield of 79.8 gm and 8.3 unit/mg tyrosinase activity in mature stage followed by mushroom grown on 2% artificial calcium carbonate at mature stage has 5 fruit bodies with yield of 53 gm and 4.03 unit/mg tyrosinase activity and 18.24 mg/100gm dry weight followed by 2.5 and 3% (of both precipitated and artificial) calcium carbonate used for mushroom growth details of which is given in Fig. 2 and 4

Calcium Contents

Calcium content was found to be maximum in *Pleurotus florida* grown on 2% precipitated calcium carbonate which was 27.8 mg/100gm dry weight at mature stage followed by 2% calcium carbonate at pre mature and mature stage. Calcium content is much higher in mushroom grown using precipitated calcium carbonate as compared to mushroom grown using artificial calcium carbonate this is also illustrated in box plot analysis (Fig. 3 and 5).

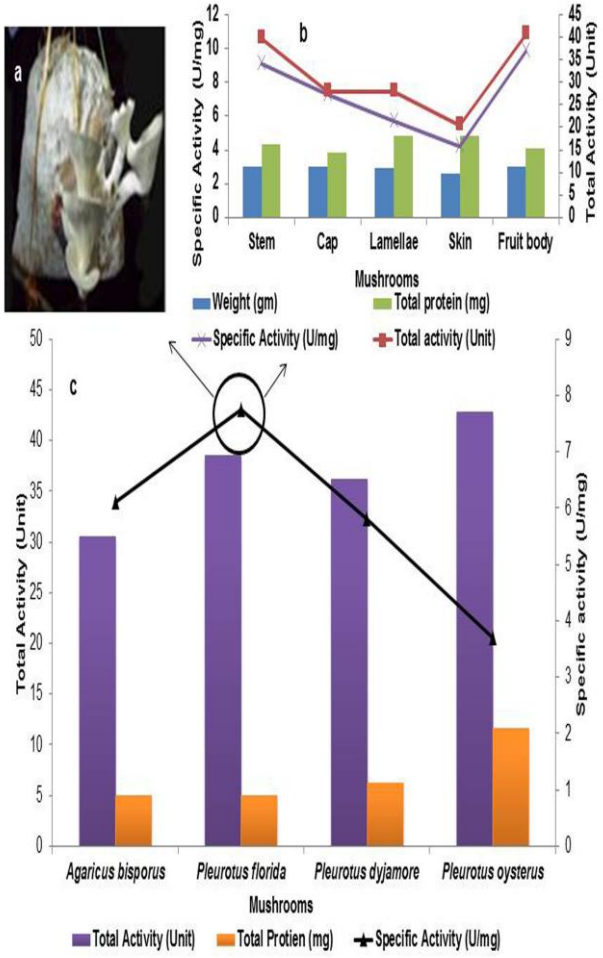

Fig. 2 Assessment of tyrosinase in Mushroom(s) Qualitative screening was done by dopachrome imagining method and Quantitative estimation was done spectrophotometrically. **a)** Appearance of fruiting body of *Pleurotus florida* grown using wheat straw as a substrate after 13 days. **b)** Various morphological parts of mushroom (*Pleurotus florida*) screened for tyrosinase activity **c)** Summary of specific activity of tyrosinase extracted from different mushroom. 1 unit of tyrosinase was defined as the amount of enzyme catalyzing the oxidation of 1 µ mol L-DOPA to dopachrome per mol

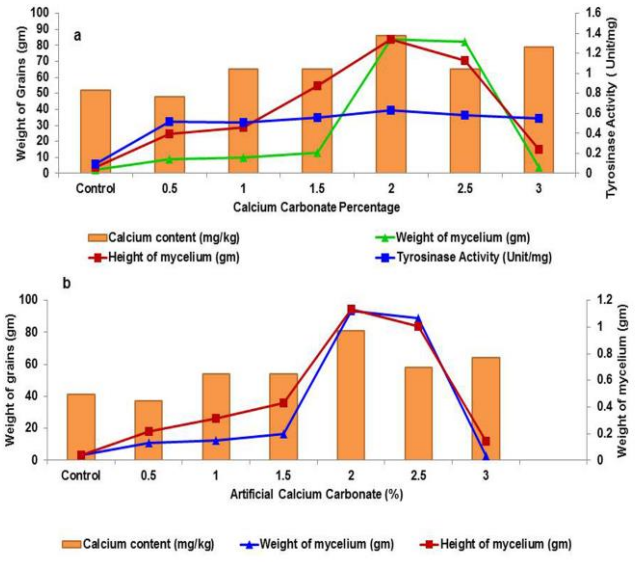

Fig. 3 Optimization of calcium carbonate for *Pleurotus florida* mycelium production **a)**Summary of mycelium grown using various concentration (0.5%, 1%, 1.5%, 2%, 2.5% and 3%) of precipitated calcium carbonate **b)** Summary of mycelium grown using various concentration of artificial calcium carbonate, both of above condition revealed highest activity with 2% $CaCO_3$

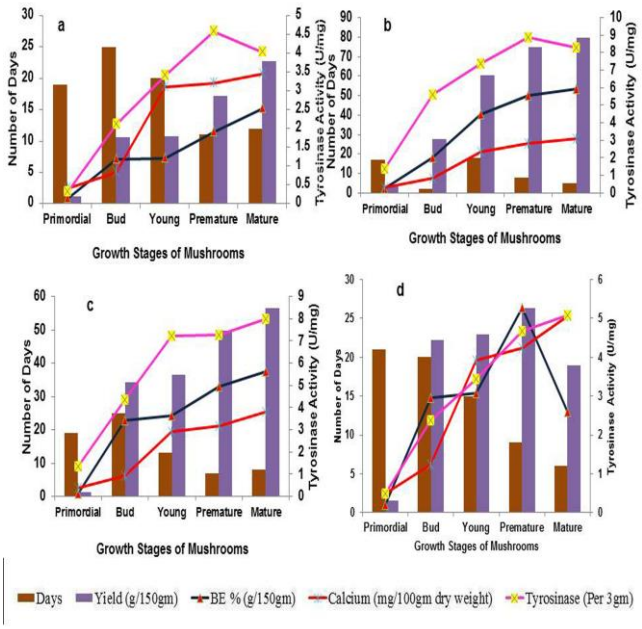

Fig. 4 Summary of biomimetically precipitated calcium carbonate utilization for growth of *Pleurotus florida* Various concentration of precipitated calcium carbonate, produced by CO_2 Sequestration by carbonic anhydrase of *Paenibacillus dendritiformis* was used for assessment of biological efficiency (BE), yield, tyrosinase activity and calcium content revealing maximum growth at premature stage. This shows the positive correlation of yield with calcium content and tyrosinase activity

D. Phenol removal efficiency of tyrosinase extracted from mushroom produced using precipitated and artificial calcium carbonate

Removal of phenol (50 mg/l) catalyzed by mushroom (produced using 2% precipitated calcium carbonate) tyrosinase extracted at different developmental stages revealed 90% phenol removal efficiency which is much higher as compared to other concentration of precipitated calcium carbonate used (Figure 3) were as mushroom (produced using 2% artificial calcium carbonate) tyrosinase extracted at different developmental revealed that tyrosinase of premature stage has 86% phenol removal efficiency which is much higher as compared to other concentration of artificial calcium carbonate used (Fig. 4).

E. Statistical Analysis and Chromatographic validation

Overall yields for mushroom crop I (grown using precipitated calcium carbonate) and Crops II (grown using artificial calcium carbonate) were compared in terms of calcium content and tyrosinase activity through principal component analysis (fig. 5). For Crop I, mushroom yields were highest with substrates supplemented with 2% $CaCO_3$ followed by 2.5% and 3%. Results for Crop II were similar to Crop I where the trend for mushroom yields for the substrate containing 2% $CaCO_3$ gave higher yields. However, yields for the non-supplemented were used as control treatment.

The product of phenol conversion has been identified as catechol in the chromatogram generated by gas chromatography with high resolution mass spectrophotometry shown in Fig. 6. Interestingly, while the peak for phenol was also gradually diminished, in the vicinity of the phenol peak a group of peaks emerged, which actually went up at the beginning and were then diminished as well during the time. Analysis by tracking the conversion of each phenol with GC confirms that these peaks were generated from the degradation of phenol. Indeed, catechol, the immediate oxidation product of phenol, appeared in GC. This suggests that those unexpected peaks referred to catechol and its further oxidizing intermediate products which were soluble. The later decline of the unexpected peaks results from the further conversion of these soluble intermediates to insoluble precipitates, which were eventually filtered off. The application of the lower purity enzyme to wastewater treatment may be advantageous due to its improved performance and lower cost. Experimental data obtained by box plot analysis and interaction between variable showed similar correlations as above analysis and thus corroborate with the results obtained by optimization studies.

IV. DISCUSSION

CO_2 by virtue of polar nature tends to traps the atmospheric infrared, resulting in warming of the earth's surface. Thus, there is a need to trap or to convert CO_2into an environment benign form. The first objective of the present study was to investigate the feasibility of using enzymes (CA) as a biocatalyst for hydration of CO_2, as well as its precipitation in the form of calcium carbonate. Our lab since last decade have been working on carbon sequestration. This paper is dedicated in exploring both the opportunities and the challenges of

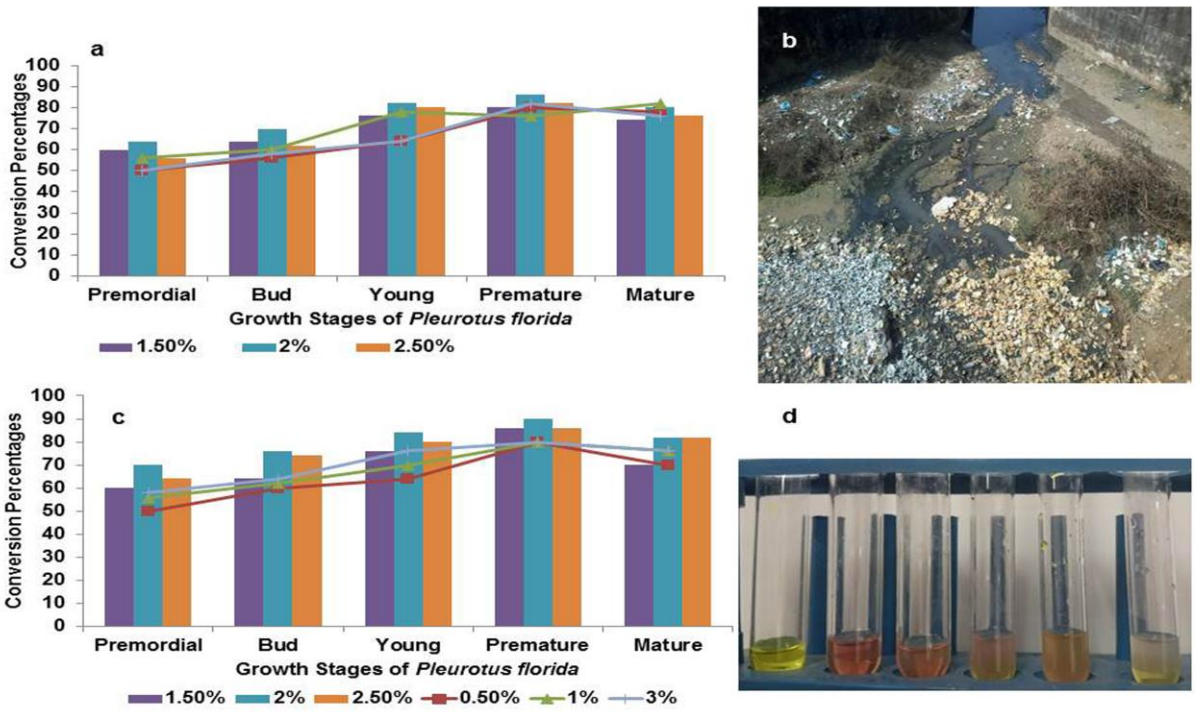

Fig. 6 Phenol degradation by various growth stages of mushroom produced using **a)** artificial calcium carbonate **c)** biomimetically produced calcium carbonate highest phenol degradation was obtained by tyrosinase from premature stage **b)** Phenolic industrial effluent collection site, Richai Jabalpur **d)** Degradation of phenol with time reduces the color intensity.

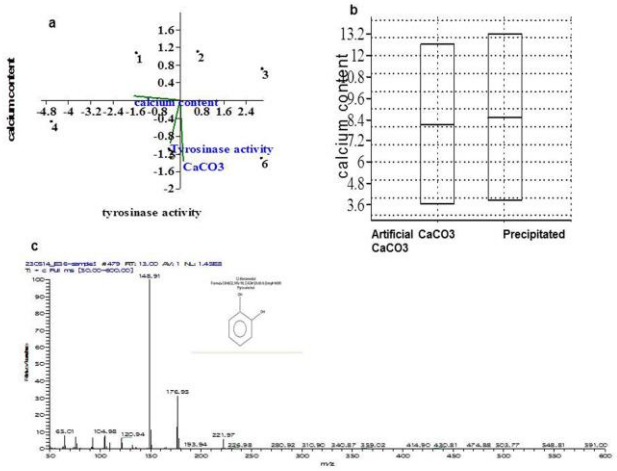

Fig. 5 Statistical and chromatographic analysis of experimental data
a) Interaction of calcium content – tyrosinase studied PCA. Positive correlation between amount of calcium carbonate, tyrosinase activity and calcium content in Pleurotus. Most values have positive effect symbolizing increase in calcium carbonate concentration can increase calcium content and tyrosinase activity. **b)** *Summary of calcium carbonate utilization in production of Pleurotus florida* Graphical data analysis by box plot technique determining the difference between the form and amount of calcium carbonate utilized by *Pleurotus florida* production through 1- factor model. Vertical axis shows amount of responsible variable (calcium content) horizontal axis showing the difference in artificial and precipitated calcium carbonate level identification. **c)** Identification of product formed by phenol degradation by GC-MS. Chromatogram of isolated compound and its credentials as catechol. Degradation of phenol was assessed using mushroom tyrosinase.

applying biological systems in Carbon Capture and Storage. We have examined prominent carbon fixation by microorganisms using carbonic anhydrase, leading to bio-based products (calcium carbonate) formation. Subsequently, we have worked in enabling novel technology for CO_2 storage and calcium carbonate utilization for mushroom production. We also surveyed phenol degradation by tyrosinase extracted by various stages of mushroom growth. The current scenario linked with elevated CO_2 concentration has inclined the development of novel strategies to mitigate the problem of global warming. The results of the first part of research plan indicated that the extracellular CA from *P. dendritiformis* is fastest among all examined during study and is much higher than *P.fragi* as reported by.Sharma and Bhattacharya [1]. Thus extracellular CA in *P. dendritiformis* is envisaged to play an important role in improving the efficiency of bicarbonate ion transport with evaluation of carbonated material as 27.33 mg of $CaCO_3$/mg enzyme with 73.2% calcium utilization.

Application of an effective enzyme system operating at low mass (protein) values under process parameters was open up for a new avenue for cost effective sequestration of CO_2 into $CaCO_3$ in an onsite scrubber. In the present study we have successfully demonstrated the sequestration of CO_2 into $CaCO_3$ using indigenous CA from *Paenibacillus dendritiformis, Oceanobacillus, Bacillus licheniformis and Pseudomonas fragi.* Sharma et al. in 2008 for the first time reported the potent CA that would be beneficial in the long-term goal of development of an efficient bioreactor for carbon-dioxide sequestration..Sharma [3]investigated that CA plays the important role in hydration reaction corresponding to calcium carbonate formation, calcite being the dominant form.

Thus, the first aim of this study was to screen bacterial isolates for the presence of Carbonic anhydrase that would help in establishing CA as an efficient biochemical marker for

carbon sequestration and environmental amelioration in the current global warming scenario linked with elevated CO_2 concentration. The present study indicates that calcium estimation can be used as an effective method for determination of sequestration efficiency with highest calcium utilization at 45°C by *P. dendritiformis* (73.2%). Calcium carbonate thus produced would be required to dispose of in a way that would avoid the leakage of CO_2 back into environment. Another application like its utilization in paint industry and other commercial application would lead to leaking of CO_2 back into environment. Capture of CO_2 in the form of calcium carbonate if not dispensed properly would be prone to emit CO_2 back into atmosphere. Thus it was inevitable to design an ambient model that utilizes CO_2 completely. Thus a novel idea is required for the utilization of precipitated calcium carbonate in the environmentally benign way so that the trapped CO_2 is not only utilization but could be used in a fruitful way.

For the first time utilization of this precipitated calcium carbonate was done for mushroom production ensuring environment friendly disposal of CO_2 from the atmosphere in the form of calcium carbonate. This novel approach conceptualization shall set path breaking way for complete carbon sequestration. The enzymatic conversion of phenolic to quinones and subsequently to humus ensures permanent sequestration of CO_2 for decades. This breakthrough thus opens new horizon in the area of carbon sequestration by virtue of fact that end product of carbon sequestration is not only environmentally benign product of carbonate but is stable also. This result is of substantial significance as for the first time application of an amalgamation of indigenous calcium carbonate at various concentration produced biomimetically from CA have been successfully used to demonstrate mushroom production.

Many agricultural by-products and waste materials have been used to produce edible-medicinal mushrooms such as oyster but variation in amount of calcium carbonate is less reported however, no data on use of biomimetically precipitated calcium carbonate for mushroom is reported. No work has been reported on the use of such calcium carbonate for the cultivation of this medicinal mushroom. Optimization of any substrate/ support material for the production process depends upon several factors such as cost and availability, but this work depends on the utilization of biomimetically produced calcium carbonate. Cultivation of edible mushrooms might be the only current process that combines the production of protein-rich food with the reduction of environmental pollution [27]. Large volumes of wheat straw are produced as agricultural by-products. Currently, wheat straw and/or rice straw are disposed off through open-field burning, which leads to serious environment pollution problem. It can support the growth of oyster mushroom, and would be one of the solutions to transform these inedible wastes into an accepted edible biomass of high market value, and serve as a cheap source of substrate for mushroom grower [28]. Therefore wheat straw is used as substrate in this research plan and has resulted in good yield of mushroom at every stage. These all reports along with our study support the use of wheat straw for the growth of *Pleurotus florida*

Among the additives, calcium carbonate found superior in enhancing the radial mycelial growth (5.7 days) of the fungus. The fast mycelial growth and basidiocarp formation due to incorporation of additive $CaCO_3$ might be due to the neutral pH of the substrate and prevention of substrate aggregation. Moreover, this additive provides sulphur and calcium which is an essential mineral nutrient for the growth of mushroom[29].

Our study has concluded that addition of calcium carbonate has increased the mushroom production at almost all the growth stages of mushroom. Addition of 2 and 2.5% calcium carbonate showed maximum mushroom growth.

In the present study, yield and mushroom size were measured in relation to its supplementation with calcium carbonate. These outcomes are supported by previous research which shows that calcium concentrations found in the apical regions of growing hyphae are higher than those found in the distal portions of hyphae. Calcium ions, therefore, play important roles in the regulation of the growth of hyphal apices and the formation of branches. Calcium also could have a role as second messenger. Calcium is believed to transduce stimuli at the cell surface that may include chemical, electrical or physical signals into specific intracellular effects. The stimulatory effect of $CaCO_3$ for mushroom yield, therefore, may be related to the various gradients and stimuli created at the hyphal tips that ultimately influence growth and development of the mushroom. It is known that $CaCO_3$ has a profound effect on pH. In addition, the release of Calcium ions from $CaCO_3$ in the substrate may be facilitated more than $CaSO_4$ by the mycelium of mushroom [21].

Researchers have demonstrated that an increase in the number and dry weight of mushrooms formed on synthetic medium containing 0.2% $CaCO_3$. However, the work of Ishikawa [30] only assessed the effect of $CaCO_3$ added to synthetic medium on first flush production of shiitake and, overall supplement levels were low (only 18% rice bran)[21]. The purpose of this work, therefore, was to determine the effect of various levels of precipitated $CaCO_3$ added to nutrient-rich synthetic medium on mushroom yield and size over a three-break production period along with its effect on tyrosinase activity. These results were analyzed using principal component analysis which is a useful statistical technique which has found application in the reduction of the original variable (PC) in order to reveal the interrelation between the different variables and to find the optimum number of extracted principle component viz. calcium carbonate. The first component (calcium carbonate) describes sample variation and the following component (tyrosinase content) shows smaller part of original variance. This means that correlated variables are explained by the same component and less correlated variables by different components. For precipitated calcium carbonate first factor represent 93% variance while for artificial it was 86%.

Various growth stages of mushroom was screened for tyrosinase activity revealing that maximum tyrosinase activity was found at stalk region the study was also supported by. Buffey [31]. Reason for the same could be that the cytoskeleton, microtubule associated proteins and actin-binding

proteins are all regulated by Ca^{2+} ions. Thus one of the main functions of Ca^{2+} is the regulation of the cytoskeleton network.

Regaladot [6] explained that the Ca^{2+} status at the tip may be responsible for the apical accumulation of vesicles and for an increase in osmotic pressure accompanied by the contraction of the cytoplasm and its migration. This strengthens the our idea that tip growth should be understood in terms of concomitant effects, resulting from a series of favorable conditions that have as their final goal the extension of a cell in a polarized manner.

Tyrosinase thus extracted from different growth stages of mushroom grown using different concentration of precipitated calcium carbonate revealed that maximum enzyme production was with 2% followed by 2.5 and 1.5 % of calcium carbonate utilization for mushroom production. Thus our result also suggests that calcium can induce the growth of Pleurotus enhancing the growth at fungal tip level. Since tyrosinase in our study was found maximum in stalk region of mushroom. Thus enhanced growth of mushroom could lead to enhanced rate of tyrosinase present in Pleurotus. Thus our work was aimed to detect the amount of calcium present in the form of calcim carbonate could enhance tyrosinase activity with respect to mushroom growth. Our result was also supported by 31. Buffey et al. 1993 who reported that calcium is evident to show positive role in cyclic AMP- stimulated tyrosinase activity.

Tyrosinases are omnipresent in nature and are considered one of the fundamental enzymes involved in several biological functions and defense mechanism [32;33].

In the recent past an enzymatic approach has attracted much interest. Thus, the present study is based on utilization of tyrosinase for phenol degradation. The reason for the above could be that enzymes could be used to oxidize phenols to free radicals or to quinones and benzoquinoneimine. The use of tyrosinase is proposed as a cheaper alternative to horseradish peroxidase because it uses molecular oxygen as an oxidant instead of expensive hydrogen peroxide [34]. Tyrosinase from mushroom grown with 2% calcium carbonate shows maximum phenol degradation, reason for which could be the maximum growth and has resulted in more tyrosinase activity and mushroom premature stage has maximum phenol degradation ~ 90% as crude extract from premature stage has maximum tyrosinase content. This study was supported by various other reports which show that in basidiomycetes maximum extracellular enzyme activity was observed in preature phase. The present research plan was thus focused to the application of tyrosinase for phenol removal 50 mg/L of phenol was removed by tyrosinase in 3 H. The present research plan emphasizes on the use of enzyme in the bioremediation of phenolic compound. Studies have shown enzymatic wastewater treatment to be feasible options for biodegradation of phenols through biological route (Fig. 6).

Earlier few reports are available on degradation of phenol but the efficiency of tyrosinase from premature phase of Pleurotus has shown higher efficiency for phenol degradation. Atlow [35] used soluble tyrosinase to remove phenol from an aqueous synthetic waste solution; up to 99% conversion of the phenol was obtained, although, at higher concentrations (1.0 g/L), conversion was limited by inactivation of tyrosinase, likely by quinines formed during the reaction [36].

End product of phenol degradation was identified as catechol through GC-MS. This product could be used to detect the pathway of phenol catabolism. Formation of catechol in early stage of phenol degradation and later on formation of muconic acid shows that tyrosinase has degraded phenol via ortho-cleavage pathway. Similar analysis was reported by Tuah [37] who has showed that the indigenous phenol metabolize phenol via ortho-cleavage pathway. The reason for these result may be the first reaction in phenol degradation is catalyzed by phenol hydroxylase whereby one oxygen atom of molecular oxygen into the aromatic ring to form catechol as the central intermediate.

V. CONCLUSION

Analysis of the experimental data obtained, concluded that CA isolated from three different microorganisms has the prospective for carbon dioxide sequestration at a rate faster for conversion of CO_2 into calcium carbonate compared to other conventional methods. CA from *Pseudomonas fragi* was found to have potential application for Biomimetic CO_2 sequestration. The CO_2 sequestration potential and reusability of CA compared to other method provides this system an immense advantage and towering edge along with economic relevance and commercial utility. The study fulfill the need to devise a competent carbon sequestration strategy that should not be based on interim goals but provide an enduring solution based on rational strategies. The principle of Biomimetic approach has been proved beyond doubt an efficient and ecofriendly process it suffers a drawback in terms of product (calcium carbonate) disposal. Thus the present study offers an environment friendly way to utilize $CaCO_3$, in mushroom production thus resulting in a value added product without the risk of CO_2 leakage back to the environment. The application of *Pleurotus florida* in bioremediation is relatively economical because the fungi Is able grown on an inexpensive agricultural waste i.e. wheat straw. In the quest, for economical and ecologically sound methods for environmental remediation, the use of mushrooms is a very good approach and solution the present study was also undertaken to probe the influence of calcium on growth and tyrosinase concentration in various growth phases of *Pleurotus florida*. The result of the experiment showed that $CaCO_3$ (2.5%) amended medium recorded high level of tyrosinase production compared to other additives. Study for the first time demonstrate A positive correlation between the biomass production and tyrosinase production in mushroom indicate the role of calcium in enhancing the biomass by play critical roles in the actively growing mycelia of *Pleurotus florida* thus, enhancing the amount of tyrosinase in the fungal cell. The ability of tyrosinase in the detection and removal of phenols has probably leaded to increased demands for tyrosinase. This work has demonstrated the feasibility and effectiveness of using tyrosinase catalyst for the treatment of phenol in water.

This new methodology is more practical and economically viable than those generally used chemical and physical methods to store and utilize atmospheric CO_2. This is the

complete cyclic method to utilize atmospheric carbon to finally convert another toxic material to humus that eventually increases fertility to soil.

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