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IN-VITRO OPTIMIZATION AND BIOTECHNOLOGICAL ASSESSMENT OF MUTANTS, HYBRID AND WILD STRAINS OF TWO WHITE-ROT FUNGUS, *LENTINUS SUBNUDUS* AND *L. EDODES* CULTURED BY SUBMERGE FERMENTATION (SMF)

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ABSTRACT

Lentinus subnudus was picked in the wild during the rainy season in the month of June and then transferred aseptically onto Potato Dextrose Agar (PDA) slant by tissue culture protocol. The optimal culture conditions for biomass and exopolysaccharide production was investigated. Growth requirements of the fungus were optimized for carbon, nitrogen, carbon-nitrogen ratio, seed culture volume and initial pH. Temperature condition was maintained at 25^{o}C at 100 rpm for 5 days. Each of the culture parameters investigated gave significant increament on biomass and exopolysaccharide production. For C-optimization, the highest biomass and exopolysaccharide was produced by starch (6.56gl^{-1}, 1.84gl^{-1}) followed by glucose (2.01gl^{-1}, 3.28gl^{-1}) respectively. Tryptone and peptone N_2-sources gave the highest biomass and exopolysaccharides as 2.44gl^{-1} and 3.06gl^{-1} respectively. The optimal C:N ratio for biomass and exopolysaccharides was found to be 1:⅓ (2.75gl^{-1}) and 1:1/5 (5.26gl^{-1}) respectively. Highest biomass and exopolysaccharides were also produced at pH 8.5 (1.90 g^{l-1}) and pH 5.5 (2.00g^{l-1}) respectively. There was no significant difference in the biomass and exopolysaccharides produced when different volume of the substrate was used. Mutants and hybrid strains of *L. subnudus* were produced by exposure to ultraviolet radiation and by crossbreeding with *L. edodes* respectively. Three (3) of the mutants that gave higher yield and better performance were selected for further biochemical analyses and then compared with the wild and hybrid varieties. Biochemical analyses such as total protein, total sugar, total phenol, total ascorbic acid content, reducing and non-reducing sugar, starch contents, anti-oxidant capacities of the wild, mutants and hybrid were performed. From our result, the mutants gave higher production and performance as compared to the wild and hybrid strains.

Keywords*:* optimization, biomass, exopolysaccharides, *Lentinus subnudus, L. edodes*

INTRODUCTION

Mushrooms are the fruit bodies of macroscopic, filamentous and epigeal fungi made up of hyphae which form interwoven web of tissue known as mycelium in the substrate upon which the fungus feeds; most often their mycelia are buried in the soil around the root of trees, beneath leaf litters, in the tissue of a tree trunk or in other nourishing substrate (Ramsbottom, 1989; Wilkinson and Buezaeki, 1982). Mushrooms belong to the class basidiomycetes in the order Agaricales whose fleshy fruit bodies and hymenia are borne on gills. They are ubiquitous group of fungi with many uses. They appear in traditional art work in form of drawings on textile material (Adenle, 1985).

Several authors like Fasidi and Kadiri, (1990a and 1993), Zoberi (1972 and 1973), Kadiri (1991), Oso (1975) and Nicholson, (1989) have shown in their various works that edible mushrooms are rich in ascorbic acid, amino acids, protein, minerals, glycogen, sugar and that protein is their most abundant nutrient. Also, mushrooms are sought for because of their toughness, meaty taste, desirable flavour and medicinal value. They are used extensively, as food item and for medicinal purposes (Oso, 1977., Chu *et al.,* 2002., Akpaja, *et al.,* 2003., Chiroro, 2004., Okhuoya, *et al.,* 2005) and due to their high economic value and the enzymes they produce.

Lentinus subnudus also known *as L. squarrosulus* is an highly-prized Nigeria white-rot mushroom, which can be picked in the wild during the rainy season between the month of April - August. It could be easily identified by its tough texture of matured sporophores, velvety stipe and funnel shaped whitish pileus (Jonathan, 2002) and belong to the group of the Basidiomycetes (Noda, 1998); family: Polyporaceae and Order: Polyporales.

L. subnudus and other varieties of mushrooms are known to grow on a wide variety of substrates and habitats. Many species of *Lentinus* have been reported to live in nature and can be cultivated on special substrate and can be cultivated on pasteurized substances (Morias *et al*., 2000; Philippousis *et al.,* 2001). Moreover, *L subnudus* have been successfully cultivated on cassava peels, rice straw, Andropogon straw, hard wood species such as *Chlorophora excelsa*, *Spondias mombin*, *Terminalia ivorensis*, and *T. superba* (Kadiri and Arzai, 2004; Adesina, *et al*., 2011). Optimization of industrial mushroom production depends on improving the culture process (Larraya, *et al*., 2003). A range of parameters including temperature, light, carbon dioxide concentration, humidity and pH have been shown to influence carpophore production Wessels, *et al*., 1987). Fruiting may also be stimulated by mechanical injury and chemical treatments (Hibbett, *et al.*, 1994.). The effect of factors such as spawn grain, culture medium, oil type and rate on the culture of *Psathyrella atroumbonata* and *L. squarrosulus* has been reported (Nwanze, *et al*., 2004a, Nwanze, *et al*., 2005a; Nwanze, *et al*., 2005b). In our research, mutants and hybrid of *L. subnudus* were produced by exposure to ultraviolet radiation (= 280nm) and crossbreeding with *L. edodes* respectively. Optimal culture conditions for the wild were investigated while biochemical analyses were performed for comparison of higher yield and performance among all the fungal strains.

MATERIALS AND METHODS

Organism and culture conditions

L. subnudus^{wt} wild type (SWT) was collected from green vegetation environment growing on a decaying mango log of wood of six months. The fruiting body of the plugged fungus was washed with 90% ethanol, and then dissected longitudinally through the gill with a sharp and sterile razor blade. The mycelium was picked and transferred aseptically onto a freshly prepared Potato dextrose agar (PDA) slants. It was maintained on slants by sub-culturing in every one month interval. The ambient temperature for culture of the fungus is 25-28^{o}C and it takes a minimum of 72 hours for optimum mycelia elongation. The fully grown fungus was maintained at 4^{0}C until when needed for use.

Lentinula edodes^{wt} wild type (EWT) was collected from Mushroom Research Centre, Himachal Pradesh Solan India.

Screening of standard media for higher yield and performance

Five fungal broths were used for culturing *L. subnudus*^{wt} to screen for the best media with high yield performance and productivity in

terms of biomass production, exopolysaccharide and enzymatic activities. Four out of the five different liquid media were used were supplied by Hi-Media Laboratory Private Ltd. Vadhani, Ind. Est. LBS Marg, Mumbai India while the other consist of a broth medium formulation according to Kwong *et al.*, 2009. The ingredients formulation and culture conditions of each of the culture media are as stated below:

Czapek Dox Broth (CDB): Sucrose (30g/l)**,** Sodium nitrate (3.00g/l)**,** Dipotassium phosphate (1.0g/l)**,** Magnesium sulphate (0.5g/l)**,** Potassium Chloride (0.5g/l), Ferrous Sulphate (0.01g/l); pH 7.3±0.2 at 25^{o}C

Mycological Broth (MB): Papaic digest of Soyabean meal (10.0g/l)**,** Dextrose (40.0g/l)**,** pH 7.0±0.2 at 25^{o}C

Potato Dextrose Broth (PDB): Potatoe infusion (200.00g/l), Dextrose (20.00g/l)**,** pH, 5.1±0.2 at 25^{o}C

Yeast Mannitol Broth (YMB): Yeast Extract (1.00g/l)**,** Mannitol (10.00g/l)**,** Dipotassium phosphate (0.50g/l)**,** Magnessium sulphate (0.20g/l)**,** Sodium chloride (0.10g/l)**,** Calcium carbonate, (1.00g/l)**,** pH 6.8±0.2 at 25^{o}C.

*Yeast Magnessium Potassium (YMK) broth***:** Glucose (20.0g/l)**,** Yeast Extract (1.0g/l)**,** $MgSO_4$. $7H_2O$ (1.0g/l)**,** KH_2PO_4 (2.0g/l)**,** pH 6.5±0.2 at 25^{o}C

Optimization of culture conditions for biomass, exopolysaccharide and enzymatic activities

The flask culture experiments were performed in a 250 ml Erlenmeyer flask containing 100 ml of the seed culture medium. The medium was inoculated with 6mm agar plug of the fungus. The following optimal culture conditions were investigated for biomass, exopolysaccharide and enzyme activities.

Carbon sources

To find a suitable carbon source for biomass, exopolysaccharide and enzymatic activity of L. subnudus various carbon sources at a concentration of 10g/l were monitored for 5 days at 25^{o}C, 100rpm on a rotary shaker incubator. The following carbon sources were used for the investigation: mannitol, starch, glucose, lactose, maltose, sucrose, fructose, mannose, galactose, arabinose, molasses and xylose. Control set-up was carbon source free in the seed culture medium.

Nitrogen Sources

To investigate the effect of different nitrogen sources on biomass, exopolysaccharide and enzymatic activity of the fungi, nitrogen compounds based on inorganic and organic nitrogen were added individually to N-source free medium at a concentration of 1g/l. Eight different N-sources were used as follows: Yeast extract, Malt extract, Beef extract, Peptone, Tryptone, $NaNO_3$, NH_4Cl, $(NH_4)_2SO_4$. The experiment was set-up for 5 days at 25^{o}C, 100rpm on a rotary shaker incubator. Nitrogen source-free medium was used as control.

Carbon-Nitrogen ratio

In order to investigate the effects of carbon-to-nitrogen ratio (C/N) ratio on mycelial growth, exopolysaccharide and enzymatic activity of Lentinus subnudus^{wt}, the concentration of the carbon and nitrogen in g/l were varied as follows: 1:0.1, 1:0.2, 1:0.25 1:0.33, 1:0.5. A carbon and nitrogen free media (0:0) was used as control.

Initial pH

To determine the optimal pH for mycelial growth, exopolysaccharide and enzymatic activity the fungus was cultivated under the initial pH ranges of 2.0 - 10.0. Experiment was carried out in 250ml Erlenmeyer flasks containing 100ml of seed culture medium on a rotary shaker incubator. The initial pH was controlled using 2 N NaOH and 2 N HCl.

Volume of seed culture

The effect of different volume of seed culture was investigated by culturing the fungus in 50 ml, 100 ml, 150 ml, 200 ml, 250 ml and 300 ml.

The 50 ml, 100 ml, 150 ml were carried out in 250 ml flask while the 250 ml and 300 ml experiment was performed in 500 ml flask.

Harvest and Quantification of Biomass and Exopolysaccharides from culture broth

(a) Biomass: The mycelia biomass was harvested from the seed culture on the 5th day of cultivation by filtering with 0.45 diameter Whatman Filter paper and the wet weight determined. The wet biomass was dried at 60^{o}C until a constant weight was obtained (Kwon et al., 2009).

(b) Exopolysaccharide (eps): Exopolysaccharides was derived from the liquid culture broth by 99% (v/v) acetone precipitation method, stirred vigorously and left overnight at 4^{o}C in a refrigerator. The precipitated crude eps was recovered by centrifugation at 10,000 rpm for 10 mins and then filtered with 0.45µm Whatman filter paper. The precipitated eps was oven dry to a constant weight and the weight of the polymer estimated.

Production of Mutants

Lentinus subnudus^{mt} mutant types (SMT**)** were produced by exposing an actively growing culture (5days old) of the fungus on Potato Dextrose Agar (PDA) plate to an ultraviolet-radiation (= 280nm) at different time durations of 15 minutes interval for 4 hrs. Mycelia plugs obtained from the culture were transferred onto the centre of a fresh PDA plates, incubated at 25^{o}C.

Production of Hybrid by crossbreeding

L. subnudus^{ht} hybrid type (SHT) was produced by cross-breeding L. subnudus^{wt} (SWT) and L. edodes^{wt} (EWT) as follows: EWT was cultured on PDA plate and incubated at 25^{o}C. On the fourth day after inoculation, SWT was inoculated onto the same plate at a distance of 8mm apart with the culture conditions remaining the same. The junction where the two fungal strains intersect was picked with a sterilized cutter and then inoculated onto a freshly prepared PDA media. The new fungal strain obtained was used as the hybrid type.

Comparison and screening of mutants for higher yield performance in biomass, exopolysaccharide and protein contents

A total of eighteen (18) fungal strains were cultured in the optimized culture conditions as follows Glucose 10g/l, Yeast Extract 10g/l, K_2HPO_4 0.5 g/l, $CaCO_3$ 1.0 g/l, $MgSO_4.7H_2O$ 0.2g/l and NaCl 0.1g/l. The initial pH of the medium was adjusted to 5.5 using 2N NaOH and 2N HCl. Equal size of each fungal agar plugs kept on PDA slants were inoculated into 100 ml substrate volume, cultured for 5 days at 25^{o}C. The wet and dry weight of the biomass from each culture flask was determined as earlier discussed above. The exoploysacharides was obtained by acetone precipitation method as already mentioned. The EPS was lyophilized and the dry weight estimated. The extracellular protein was determined according to Lowry et al., (1951). SMT060, SMT-120 and SMT-135 were selected out of the eighteen mutants based on biomass, exopolysaccharides and protein production levels.

Biochemical analysis

i. Total Protein estimation

Total protein was estimated by Folin-Ciocalteau's method as modified by Lowry *et al.,* (1951) using bovine serum albumin as standard. The blue colour developed was measured at 660 nm against the blank and standard solution of protein was also run simultaneously.

ii. Enzyme Activity assay

Two extracellular lignocellulotic enzymes (Pyranose 2-oxidase and Laccase) associated with white-rot fungus were extracted and assayed accordingly as follows:

a. Determination of Pyranose 2-oxidase Activity assay

Pyranose 2- oxidase (P2OX) activity was determined using the chromogen ABTS [(2,2-

azinobis(3-ethylbenzthiazolinesulfonic acid)] ($\varepsilon 420 = 3.6 \times 10^4$ $mM^{-1}cm^{-1}$) according to the modified method of Danneel *et al*., 1993. The standard 1 ml assay mixture contains 10mM ABTS, 2U of Horseradish Peroxidase Type II Sigma, 100 µmol D-glucose, in 10 mM sodium phosphate buffer (pH 6.5), 100µl of the diluted enzyme was used. The mixture was incubated at 30°C in Water-bath B-480 (manufactured by Buchi, Switzerland) for 3mins. Oxidation of ABTS was monitored by spectrophotometer at 420nm.

b. Determination of Laccase Activity assay:

Laccase activity assay was determined according to the modified method of Shin and Lee, 2000; Saranyu and Rakrudee, 2007 as follows:

The routine assay for laccase was based on the oxidation of [(2,2-azinobis(3-ethylbenzthiazoline-6-sulfonate) diammonium salt] (ABTS) (Sigma) ($\varepsilon 420 = 3.6 \times 10^4$ $mM^{-1}cm^{-1}$). 50µl of the enzyme was incubated in 940µl of 0.1 M Sodium acetate buffer (pH 4.5) containing 10µl of 10mM ABTS incubated at 30°C in Water-bath B-480 for 10mins. The reaction mixture was stopped by adding 50µl of 50% (w/v) Trichloroacetic acid (TCA). Oxidation of ABTS was monitored by spectrophotometer at 420nm.

iii. Estimation of total sugar content of the exopolysaccharides

The total sugar contents (TSC) of the exopolysaccharides produced from the submerge cultures of wild, mutants and hybrid fungal strains were estimated using Anthrone method with glucose as standard, according to Sadasivam and Manickam 1992. Briefly, 100mg of the sample was weighed into a boiling tube, hydrolyzed in boiling water for 3h with 5ml of 2.5 N HCl. The sample was cooled to room temperature and then neutralized with solid sodium carbonate until effervescence ceases. The volume was made up to 100ml and then centrifuged. The supernatant was collected and 0.5 and 1 ml aliquots taken for analysis. The volume was made to 1 ml in the tubes by adding distilled water. 4 ml of anthrone reagent was added to the tubes heated for 8 minutes in a boiling water bath. The sample tubes were cooled rapidly and the green to dark green colour read at 630 nm.

iv. Estimation of starch content by Anthrone method

0.1g of the sample was weighed and homogenized in hot 80% ethanol to remove the sugars. This was centrifuged and the residue was retained. The residue was washed repeatedly with hot 80% ethanol till the washings did not give colour with anthrone reagent. The residue was dried well over a water bath. 5.0 ml of water and 6.5 ml of 52% Perchloric acid was added to the residue. This was centrifuged and the supernatant saved. The extraction process was repeated using fresh Perchloric acid and then centrifuged. The supernatant was pooled and made up to 100 ml with distilled water.1 ml aliquot of the sample was used for analysis.. 4 ml of anthrone reagent was added, heated for 8 minutes in a boiling water bath. The tubes were cooled rapidly and the intensity of green to dark green colour read at 630 nm.

v. Estimation of reducing and non-reducing sugars by DNS method

Reducing and non-reducing sugar components of the exopolysaccharides were determined using Dinitrosalicyclic acid (DNS) reagent as follows: 100mg of each sample was weighed and the sugars were extracted with 5ml of hot 80% ethanol twice. The supernatant was collected and evaporated on water bath. 10ml of water was added to dissolve the sugars. 1ml aliquot of each of the sample was taken for analysis and the volume made up to 3 ml in test-tube. Each sample was prepared in triplicate. 3ml of DNS reagent was added and mixed together. The solution was heated for 5mins in a boiling water bath. After the colour has

developed, 1ml of 40% Rochelle salt solution was added when the contents in the tube were still warm. The tubes were cooled under running tap and the absorbance was measured at 510nm. The amount of reducing sugar in each of the sample was calculated using glucose as standard.

vi. Determination of total phenols in the exopolysaccharides

Total soluble phenols were estimated using Folin-Ciocalteu reagent (FCR) with catechol as standard (Malick and Sigh 1980). The blue coloured complex was taken at 650nm against a reagent blank.

vii. Estimation of Ascorbic acid using 2, 6-dichlorophenol indole phenol titration method

5ml of the working standard was taken with a pipette into a 100ml conical flask. 10ml of 4% oxalic acid was added and this was titrated against the dye. 1g of each of the sample was extracted in 4% oxalic acid and the volume made up to 100ml. This was then centrifuged at 10,000 rpm for 15 mins. 5 ml of the supernatant was taken and 10ml of 4% oxalic acid added. This was titrated against the dye. The end point was the appearance of pink colour which persisted for a few minutes. The amount of the dye consumed is equivalent to the amount of the ascorbic acid.

viii. Determination of antioxidant activities of the exopolysaccharides.

Methanol and dichloromethane extractions were used for determining hydrophilic and lipophylic antioxidant activities of the exoploysaccharides (Arnao et al., 2001).

Sample Extraction

(a) Methanol extraction

1g of the eps was mixed with 10ml methanol and then homogenized using vortex. The homogenate were kept at 4^oC for 12 hours and then centrifuged at 10,000 rpm for 20 mins. The supernatants were recovered and stored at -20^oC until analysis.

(b) Dichloromethane extraction

The pellet obtained from the methanol extract was re-dissolved with 10ml dichloromethane, and homogenized for anti-oxidant activity. The homogeneate was centrifuged at 10,000 rpm for 20 mins. The supernatants were recovered and stored at -8^oC until further analysis. Briefly, 150µl of each of the extract for each sample was mixed with 2850 µl of the ABTS. It was kept for 2 hours in a dark condition. Absorbance was taken at 734nm using spectrophotometer. The standard curve was obtained using Trolox and results were expressed in µM Trolox Equivalent (TE)/g fresh weight.

Statistics

Analysis of the data was performed by one-way analysis of variance (ANOVA), One-Sample t-test and Karl Pearson Correlation coefficient was determined. Differences at $P < 0.05$ were considered to be significant. Analysis was done using Ms Excel Sheet 2007, Origin Lab 8 and Graph Pad Prism 5 statistical softwares. Each experiment was repeated three times with three replicates each.

RESULTS AND DISCUSSION

Submerge culture of *L. subnudus*^{wt} (SWT) in different standard media

Our first attempt was to culture SWT in five different media by sub-merge fermentation for biomass, exopolysaccharide, protein production and enzyme assay. Among all the five media used, YMKB gave the highest biomass ($60\pm6.39 mgl^{-1}$) and exopolysaccharide (960 ± 10.83 mgl^{-1}) production and least in CDB which yielded 19.50 ± 4.58 mgl^{-1} and 100 ± 20.53 mgl^{-1} biomass and exopolysaccharide respectively, although higher exopolysaccharide was also recorded by MB as shown in Table 1. Total protein estimated was highest in YMB (1.734 ± 0.93 $mgml^{-1}$) and least in PDB (0.186 ± 0.05 $mgml^{-1}$). There were significant differences ($P < 0.05$) between the biomass,

exopolysaccharide and protein produced among the five broth media used. Previous researchers already reported that submerge culture obviously give rise to potential advantages of higher mycelial production in a compact space and shorter time without significant problem of contamination (Bae *et al*., 2000; Park *et al*., 2001; Sinha *et al*., 2001). Enzyme assay done showed highest laccase and Pyranose 2-oxidase activity in CDB and YMKB as 13.345 $Uml^{-1}min^{-1}$ and 12.87 $Uml^{-1}min^{-1}$ respectively (Table 1).

Effect of different carbon sources

In order to determine the suitable carbon source for higher biomass and exopolysaccharide production, SWT was cultured in broth media, each containing various carbon sources. Each of the carbon sources was added to the synthetic media at a concentration of 1% (w/v) and then cultured for five days. The fungus showed a good growth response to most of the carbon sources as compared to the control experiment in which no carbon source was added. As shown in Fig. 1, starch gave the highest biomass production ($6.56gl^{-1}$) followed by dextrose ($2.01gl^{-1}$) among all the twelve carbon sources investigated. This is contrary to previous report on the optimization for mycelial biomass and exopolysaccharide production in submerge cultures of *Cordyceps militaris* (Jeong, *et al*., 2009). For exopolysaccharide production, the highest yield was recorded in molasses ($3.96\ gl^{-1}$), followed by maltose ($3.68\ gl^{-1}$) as carbon sources. Although, glucose has already been reported by some previous investigators as a good carbon source for biomass and exopolysaccharides production in submerge cultures (Xu *et al*., 2003; Nour El-Dein *et al*., 2004; En-Shyh and Shu-Chiao, 2006), our result shows that *L. subnudus* was able to utilize both monosaccharides and disaccharides sugar compounds for biomass and exopolysaccharides production. This result is in accordance with nutritional requirement for biomass and exopolysaccharide production in several mushrooms (Sudhakaran and Shewale 1988; Fang and Zhong, 2002b; Ji-Hoon *et al*., 2004; Jeong, *et al*., 2009). Lactose and arabinose gave the lowest biomass and eps yield as $0.15\ gl^{-1}$ and $0.16\ gl^{-1}$ respectively. This result is in consonance with that of previously reported investigators where arabinose gave lowest biomass yield (Madunagu 1988, Jonathan and Fasidi, 2001), although contrary to report by Gbolagade *et al* (2006). In order to further understand the relationship between biomass and exopolysaccharide produced as a result of various carbon sources used, Pearson coefficient of correlation (r) was worked out. The outcome shows that there is no correlation between biomass and exopolysaccharide ($r = -0.087$), suggesting that, both products are independent of each other although there was significant difference among the values obtained ($P < 0.05$). We also observed in our results that mushrooms may show different growth responses to carbohydrate utilization as compared to results already reported by other researchers.

Effects of different nitrogen sources

To investigate the effect of different nitrogen sources on biomass and exopolysaccharides production in submerge culture of *L. subnudus*, eight nitrogen sources were used consisting of five organic and three inorganic nitrogen sources. The basal medium was supplemented with the nitrogen sources at a concentration of 0.1% (w/v). A nitrogen limited control was also set-up. Among the nitrogen sources tested, tryptone gave the highest biomass production ($2.44gl^{-1}$), followed by ammonium sulphate ($1.36gl^{-1}$) and ammonium chloride ($1.30gl^{-1}$) as shown in Fig. 2. Moderate biomass yield were recorded in beef and yeast extract, peptone and sodium nitrate with the lowest in meat extract ($0.045gl^{-1}$). For eps production, peptone gave the highest yield ($3.06gl^{-1}$), followed by ammonium

chloride (2.66gl^{-1}), with moderate yields in beef, meat and yeast extract. Reduced production of exopolysaccharide was recorded for both sodium nitrate and ammonium sulphate, whereas, tryptone gave the lowest yield (0.62gl^{-1}). This means that a nitrogen source used to improve biomass production may not necessarily support a high yield of exopolysaccharides in *L. subnudus.* Our result reveals that organic nitrogen sources were favourable in biomass and exopolysaccharide production in sub-merge culture of *L. subnudus* as compared to inorganic sources. The result of which is in agreement with previous report on biomass and exopolysaccharide production in submerge fermentation of *Sarcodon aspratus* (Ji-Hoon, 2004), and *L. tuberregium* (Manjunathan and Kaviyarasan, 2011). Pearson correlation coefficient (r = 0.0084) shows that there was a low degree of correlation between biomass and exopolysaccharide produced when different Nitrogen source were used whereas on the other way round, there was a significant difference between the means of the values obtained ($p<0.05$).

Effect of carbon-nitrogen ratio

In order to determine the effects of carbon-nitrogen ration (C/N) on biomass and exopolysaccharide production, of *L. subnudus*, the concentration of dextrose and yeast extract in the basal medium was varied. Concentration of 1% (w/v) of each of dextrose and yeast extract in the basal medium was used for ratio 1:1. Other ratios were varied accordingly as follows: 1: 0.1, 1: 0.2, 1: 0.25 1: 0.33, 1: 0.5. Highest increase in biomass was recorded in C/N ratio 1: 0.3 (2.75 gl^{-1}) and least in 1: 0.1 (0.33 gl^{-1}) as compared to the control without carbon and nitrogen sources (Fig. 3). The trend in the result shows that there was increase in biomass production as the C/N ratio increases up to a point after which further increase in nitrogen brought a decrease in biomass production. This result is the same as that reported in *L. tubberregium* (Manjunathan and Kaviyarasan, 2011). Highest exopolysaccharide was obtained in C/N ratio 1:0.2 (5.26g^{-1}), and least in 1:0.5 (0.76gl^{-1}). There was low correlation between the biomass and exopolysaccharide (r = 0.026) although significant effect was observed at $P < 0.05$

Effect of initial pH

Among the environmental factors, culture pH has been known to significantly influence cellular morphology and metabolites biosynthesis (Heald and Kristiansen 1985; Salleh *et al*., 2008). To determine the optimal pH for biomass and exopolysaccharides production, *L. subnudus* was cultivated under different initial pH (2-10) in shake flask culture. Figure 4 shows that the effects of initial pH on biomass and exopolysaccharide production were variable. In these experiments, high biomass was obtained at pH 6.5 (1.79gl^{-1}) and highest at pH 8.5 (1.9 gl^{-1}). For eps production, high amount was recorded at pH 6.5 (1.96gl^{-1}) and maximum at pH 5.5 (2.00 g^{l-1}). This shows that *L. subnudus* can grow optimally either at sub-acidic and sub-basic culture medium. This result is in alignment with result already reported for biomass production in submerge cultures of *C. militaris* (Jeong *et a*l., 2009). Jonathan (2002) already suggested that good growth of mushrooms could be obtained at moderately or slightly acidic pH. At lower and higher value of pH, biomass and eps obtained declined- the result of which suggest that strongly acidic and highly alkaline culture conditions were unfavourable to biomass and eps production in submerge cultures of *L. subnudus*. On the other hand, some other workers have recorded optimum pH 5.5 for other exopolysaccharide synthesizing fungi such as *Pleurotus pulmonarius* (Nour El-Dein *et al*., 2004). Further more, it has also been reported that many kinds of ascomycetes and basidiomycetes exhibit more acidic pH optima during submerge culturing

(Yang and Liau, 1998a,b; Lee *et al*., 1999; Yang *et al*., 2000a,b; Park *et al*., 2001). All of these results lead to the conclusion that there is a relationship between the degree of exopolysaccharide production, biomass and the initial pH of the culture medium. (En-Shyh Lin and Shu-Chiao Sung, 2006). There is a moderate correlation (r = 0.583) between biomass and exopolysaccharide produced as the pH of the culture medium changes. However, low correlation exists between pH and biomass (r = 0.098); pH and exopolysaccharide (r = 0.346) respectively.

Effect of seed culture volume

Fig. 5 shows the effect of seed culture volume on biomass and eps production. Equal size of *L. subnudus* agar plugs were inoculated into each of the different seed culture volume, while other culture conditions remain constant. As indicated in Fig. 5, we obtained variability in the quantitative estimate of biomass and eps produced with the highest biomass and eps obtained in the 100ml (0.095g) and 300ml (0.22g) seed culture volume respectively, although, the values were not significantly different as compared to other seed culture volume used. This suggests that, the mineral composition, and cultural conditions has a greater influence than the seed culture volume in biomass and exopolysaccharide production by *L. subnudus*. This is further established as there is was no correlation (r = -0.083) between biomass and exopolysccharide obtained.

Generation of new hybrid by cross-breeding

A new hybrid of *L subnudus* tagged as "*subnudus* hybrid type" (SHT) was produced by cross-breeding the wild strain of the fungus (SWT) with *L. edodes* wild strain (EWT) which has been reported as a very important mushroom of desirable characteristics. This is the first time we are reporting the generation of a hybrid from the two wilds in the same genus. Further biochemical analysis were done using the new strain and then compared with the wild and selected mutants.

Culture of wild, mutants and hybrid of *L. subnudus* in fungal broth

Fifteen (15) mutants and one hybrid were produced as earlier discussed. Out of the fifteen mutants, the best three (SMT-060, SMT-120 and SMT-135) with good physiological and biochemical characteristics were selected as shown in Plate 1 (a-c) and then compared with *L. subnudus*^{wt} (SWT), *L. edodes*^{wt} (EWT) and their hybrid (SHT). We cultured the five strains in the optimized medium to investigate if there would be increase or improvements in their biomass and exopolysaccharide production in submerge fermentation. It was observed that SMT-120 gave the highest biomass (2.830gl^{-1}) and exopolysaccharide (17.71 gl^{-1}) among the fugal strains with the least production obtained in SWT. Extracellular proteins extracted and quantified from submerge culture of each of the fungal strains by Lowry's method showed that total protein estimated was highest in SMT-060 (5.187 gl^{-1}) and least in SWT (0.734 gl^{-1}) as indicated in table 2. There was significant difference ($P < 0.05$) in biomass, exopolysaccharide and protein produced among all the fungal strains

Enzyme extraction and purification

The culture filtrate of each of the wild, mutants and hybrid strain were evaluated for the production of two lignolytic enzymes namely; pyranose 2 – oxidase and laccase. The crude enzymes were fractionated with $(NH_4)_2SO_4$ and enzyme assay done accordingly.

Enzyme assay and activity

Pyranose 2-oxidase: Pyranose 2 oxidase (P2OX) has already been reported to have roles in ligninolysis (Daniel *et al*., 1994; Volc *et al*., 1996), production of peroxide for peroxidases, and synthesis of the antibiotic cortalcerone (Baute and Baute, 1984). It is also of interest for numerous biotechnological applications,

analytical methods, and synthesis of novel chemicals (Giffhorn, 2000; Theodorus *et al*., 2004). P2OX activity was carried out among all the five fungal strains. EWT gave the highest activity (10.14 $Uml^{-1}min^{-1}$) and least in SMT-135 (0.821 $Uml^{-1}min^{-1}$), although activity was relatively higher in SMT-120 and SMT-060 (Table 2). Moreover, several wood decaying fungi in addition to *L. subnudus* and *L. edodes* have been reported to also produce P2OX (Giffhorn, 2000), including *Oudemansiella mucida*, *Trametes versicolor* (Daniel *et al*., 1994), *Polyporus obtusus* (Ruelius *et al*., 1968), *Phlebiopsis gigantea* (Scha¨fer *et al*., 1996), and *Trametes multicolor* (Volc *et al*., 1999). Some POX activity has also been observed in culture fluid, but this appears to be related to cell lysis (Daniel, *et al.*, 1992). P2OX can also be extracted from decayed birch (Daniel, *et al.*, 1994, Daniel, *et al.*, 1992), and an extracellular activity is observed when the fungus is grown on the natural lignocellulosic substrate such as wheat straw (Vyas *et al*., 1994)

Laccase*:* Although there are many reports dealing with extracellular Laccases produced by white-rot basidiomycetes and ascomycetous fungi (Leonowicz *et al*., 2001; Claus, 2004), in our research, attempt was made to also produce extracellular laccase from the submerge cultures of wild, mutants and hybrids of *L. subnudus* and compare their activity with one another. Besides, intracellular Laccase from fully browned gills of the fruiting body of *L. edodes* (Masaru *et a*l., 2003), *L. Panus (trigrinus)* and other white-rot fungus has also been reported (Burke and Cairney, 2002; Schlosser *et al*., 1997; Roy-Arcand and Archibald, 1991). Laccases have received much attention from researchers in last decades due to their ability to oxidise both phenolic and non-phenolic lignin related compounds as well as highly recalcitrant environmental pollutants, which makes them very useful for their application to several biotechnological processes (Susana and José, 2006). Such applications include the detoxification of industrial effluents, mostly from the paper and pulp (Kuhad *et al*., 1997), textile and petrochemical industries (Abadulla *et al.*, 2000; Blánquez *et al*., 2004; Hou *et al.*, 2004; Rodríguez Couto *et al.*, 2004a, 2005), medical diagnostics and as a bioremediation agent to clean up herbicides, pesticides and certain explosives in soil ((Durán and Esposito 2000; Pointing, 2001). Among the five fungal strains tested for laccase activity, EWT showed the highest enzyme activity (26.60 $Uml^{-1}min^{-1}$) and least activity was expressed in SMT-060 (2.16 $Uml^{-1}min^{-1}$) as shown in table 2.

Total sugar, total phenol and total ascorbic acid of the exopolysaccharide

The total sugar contents (TSC) of the exopolysaccharides produced from the submerge cultures of wild, mutants and hybrid fungal strains were estimated using Anthrone method with glucose as standard (Sadasivam and Manickam, 1992) while total phenol was estimated using Folin-Ciocalteu reagent (FCR) using catechol as standard as already explained**.** Ascorbic acid (which is a sugar acid and an antioxidant agent) in the exopolysaccharides was quantified using 2, 6-dichlorophenol indole-phenol dye method (Sadasivam and Theymoli, 1987; Heinoen *et al*., 1989). Analysis of the total sugar, total phenol and total ascorbic acid contents among the five fungal strains tested is shown in table 3. EWT showed the highest total sugar content and least in SMT-060. Total phenol was highest in SMT-135 and least in SHT while total ascorbic acid was highest in SMT-060 and least in EWT, although the values are not significantly different from one another. The presence of phenol and ascorbic acid is an indication that these fungus are promising source of antioxidant which are of natural origin as already also implicated in medicinal mushroom such as *Inonotus obliquus* (In *et al*.,

2007) and Kale plants (Imtiyaz *et al*., 2005). Phenolic compounds are known as powerful chain breaking antioxidants (Shahidi and Wanasundara, 1992). Phenols are very important plant constituents because of their scavenging ability due to their hydroxyl groups (Hatano *et al*., 1989). The phenolic compounds may contribute directly to antioxidative action (Duh *et al*., 1999). It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans, when ingested up to 1g daily from a diet rich in fruits and vegetables (Tanaka *et al*., 1998). The Folin-Ciocalteau assay has been utilized to measure polyphenols for a number of different plants, including mushrooms. A correlation has also been shown between higher antioxidant activity and larger amount of total phenolics in extracts from various commercial mushrooms (Cheung and Lee, 2000). Analysis of our results shows that there was no significant difference in the phenol produced among all the strains ($P < 0.05$), although there were significant differences in sugar, ascorbic acid and total protein ($P < 0.05$) among all the fugal strains as indicated in table 3.

Determination of reducing sugar, non-reducing sugar, amount of starch of the exopolysaccharide

Sugar were assayed using 3, 5-dinitrosalicyclic acid (DNS) which assays sugars by their reducing properties (Sadasivam and Manickam, 1992). The content of non-reducing sugars was obtained by subtracting the reducing sugars from total carbohydrate contents, while amount of starch of the exopolysaccharides were determined by the Anthrone method (Hodge and Hofreiter 1962; Thayumanavan and Sadasivam, 1984; Sadasivam and Manickam, 1992). Table 4 shows the percentage composition of the reducing, non-reducing and amount of starch in the exopolysccharides of the fungal strains. EWT gave the highest composition of the reducing and non-reducing sugars as 14.93 % and 50.40 % respectively although SMT-135 also yielded a very good composition of these sugars. Percentage starch composition was highest in SMT-060, although there was no significant difference in the percentage starch composition among the fungal strain ($P < 0.05$)

Antioxidant activity determinations in methanolic and dichloromethane extract.

Antioxidant activities in methanolic and dichloromethane extract was measured using 2,2-Azinobis-(3-ethyl) benzothiazolline)-6 sulphonic acid diammonium salt ABTS as earlier mentioned. Antioxidant activity was highest in dichlorometane extract especially in EWT and reduced in methanolic extract of SMT-060 although there was no significant differences ($P< 0.05$) among the values obtained as shown in table 5). This result is similar to previous report by researchers who reported potent antioxidant activity in *Inonotus obliquus* (In *et al*., 2007) and *Pleurotus pulmonarius* (Badole *et al*., 2008). There is considerable evidence that these antioxidants could help to prevent various diseases including myocardial and cerebral ischemia, diabetes, rheumatoid arthritis, inflammation, and cancer-initiation as well as in aging process (Coyle 1993; Margaill *et al*., 2005) because they have the capacity to quench free radicals. These natural antioxidants have advantage over some synthetic antioxidants such as butylated hydroxyanisola (BHA) and butylated hydroxytoluene (BHT), which have been reported to exerts toxicological effects as compared to natural antioxidants (Saito *et al.*, 2003; Stefanidou *et al*., 2003).

CONCLUSION

All the fungal strains gave remarkable potentials in enzymes synthesis, anti-oxidants, sugar, phenol, ascorbic acid, exopolysaccharides and proteins. Therefore, the exploitation of wild, mutants and hybrid strains of *L. subnudus* using

biotechnological tools and protocols is thereby suggested for large scale production.

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Table 1 Estimation of Biomass, exopolysaccharides and enzymatic activity of *L. subnudus* (SWT) in different basal media

Fungal strains	Biomass dry wt. (mgl^{-1})	Dry eps. (mgl^{-1})	Total protein ($mgml^{-1}$)	Laccase Act. ($Uml^{-1}min^{-1}$)	P2OX Act. ($Uml^{-1}min^{-1}$)
MB	30.50 ± 1.08^{c}	840.00 ± 2.46^{a}	1.542 ± 0.78^{a}	5.32^{b}	10.93^{b}
CDB	19.50 ± 4.58^{c}	100.00 ± 2.53^{b}	1.420 ± 0.82^{a}	13.34^{a}	10.04^{b}
PDB	42.00 ± 2.87^{c}	60.00 ± 8.41^{b}	0.186 ± 0.05^{b}	2.09^{b}	41.92^{a}
YMB	55.00 ± 4.35^{ac}	560.00 ± 8.47^{ac}	1.734 ± 0.93^{a}	2.30^{b}	4.33^{b}
YMKB	60.00 ± 6.39^{ab}	960.00 ± 10.83^{ac}	1.127 ± 0.42^{a}	5.10^{b}	12.87^{b}

Data are means of three replicates; ± values represents standard deviation of the mean; Mean followed by same letters in the same column are not significantly different ($P<0.05$) from each other according to Turkey's Multiple Comparison Test.
MB: Mycological Broth; CDB: Czapek Dox Broth; PDB: Potato Dextrose Broth; YMB: Yeast Mannitol Broth; YMKB: Yeast Magnesium-Potassium Broth.

Table 2 Estimation of Biomass, exopolysaccharides and enzymatic activity of wild, hybrid and mutants fungal strain

Fungal strains	Biomass dry wt. (mg^{l-1})	Dry eps. (mgl^{-1})	Total protein (mgl^{-1})	Laccase Act. ($Uml^{-1}min^{-1}$)	P2OX Act. ($Uml^{-1}min^{-1}$)
SWT	290.05 ± 5.12^{c}	8230 ± 10.54^{c}	0.734 ± 0.12^{cd}	10.07^{b}	4.66^{b}
EWT	510.29 ± 2.44^{c}	6530 ± 210.71^{c}	0.895 ± 0.04^{cd}	26.60^{a}	10.14^{a}
SHT	190.15 ± 1.67^{c}	6780 ± 31.42^{c}	1.962 ± 0.11^{bd}	2.16^{c}	1.42^{b}
SMT060	320.26 ± 2.95^{c}	17640 ± 30.78^{b}	5.187 ± 0.62^{a}	2.98^{c}	5.05^{b}
SMT120	2830.87 ± 1.10^{a}	17710 ± 30.17^{a}	3.189 ± 0.12^{b}	2.75^{c}	9.23^{a}
SMT135	1590.03 ± 3.07^{b}	7890 ± 23.48^{c}	$2.606\pm o.14^{b}$	3.96^{c}	0.82^{b}

Data are means of three replicates ± standard deviation of the mean. Means with different letters in the same column are significantly different ($P<0.05$) from each other according to Turkey's Multiple Comparison Test.

Table 3 Biochemical analysis of exopolysaccharides of wild mutants and hybrid of *L. subnudus*

Fungal strain	Total sugar µg/100ml	Total Phenol mg/100ml	Total ascorbic acid mg/100ml
SWT	20.00±0.44[b]	1.96±0.06[b]	39.91± 11.97[a]
EWT	65.33 ± 10.79[a]	2.56 ±0.06[b]	23.94± 0.00[a]
SHT	20.33 ± 3.97[b]	0.96± 0.02[b]	35.90 ± 0.00[a]
SMT-060	18.23 ±2.55[b]	2.04 ±0.02[b]	43.89 ±3.99[a]
SMT-120	40.33 ±3.52[b]	1.19 ±0.02[b]	35.9 ± 0.00[a]
SMT-135	46.00 ±4.30[b]	13.14 ± 2.20[a]	39.93 ±7.63[a]

Data are means of three replicates ± standard deviation of the mean. Means with different letters in the same column are significantly different (P<0.05) from each other according to Turkey's Multiple Comparison Test.

Table 4 Estimation of reducing, non-reducing sugar and starch of wild, hybrid and mutants fungal strain.

Fungal strain	Reducing sugar %	Non-reducing sugar %	Amt. of starch %
SWT	8.33 ± 0.77[a]	11.10 ± 0.77[a]	10.35 ± 0.24[a]
EWT	14.93 ± 0.24[a]	50.40 ± 0.24[b]	9.17 ± 0.73[a]
SHT	8.69 ± 0.31[a]	11.64 ± 0.31[a]	9.29 ± 1.09[a]
SMT-060	8.20 ±0.04[a]	10.03 ± 0.04[a]	10.50 ±1.83[a]
SMT-120	8.46 ±0.07[a]	31.87 ± 0.07[b]	8.13 ±1.40[a]
SMT-135	10.11 ± 0.20[a]	35.89 ± 0.20[b]	12.16 ± 1.11[a]

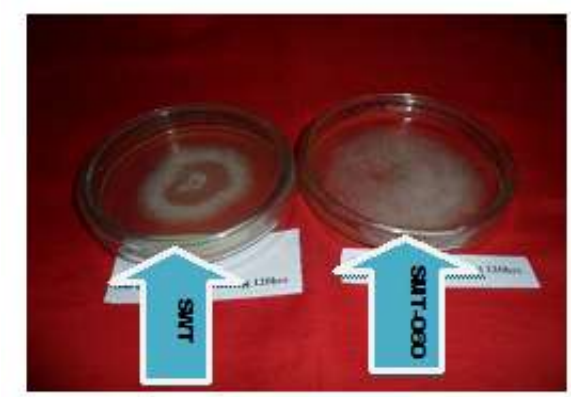
Data are means of three replicates ± standard deviation of the mean. Means with different letters in the same column are significantly different (P<0.05) from each other according to Turkey's Multiple Comparison Test

Table 5 Trolox equivalent anti-oxidnat capacitities (TEAC) of the exopolysaccharides obtained from wild, hybrid and mutants fungal strain

Fungal strain	Methanolic extract µM TE/g.fr.wt.	Dichloro-methane extract µM TE/g.fr.wt.
SWT	348.55± 4.57[a]	491.26±5.74[a]
EWT	431.55±9.20[a]	655.76 ±2.90[a]
SHT	332.05±3.82[a]	648.65 ±7.68[a]
SMT-060	143.63 ±10.94[a]	622.11 ±11.51[a]
SMT-120	329.09 ±5.08[a]	623.13±5.50[a]
SMT-135	375.54±14.42[a]	673.19±8.60[a]

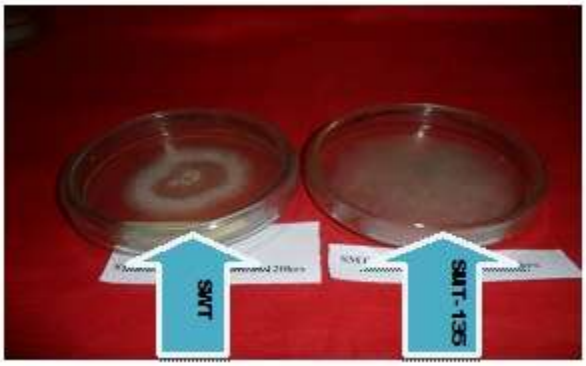
Data are means of three replicates ± standard deviation of the mean. Values with same letters in the same column are not significantly different (P<0.05) from each other according to Turkey's Multiple Comparison Test.

PLATES



(a)

(b)



(c)

Plate 1(a-c) Phenotypic morphology of wild and mutants types of *L. subnudus* cultured on solid media for 5 days

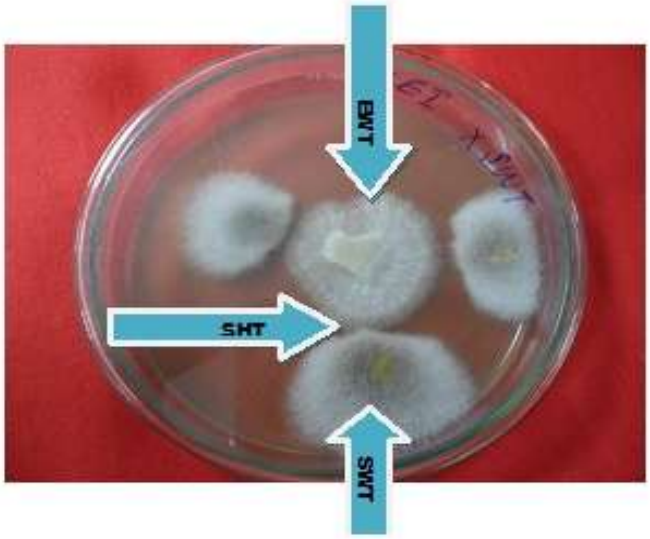


Plate 2 Production of *L. subnudus* hybrid type (SHT) by crossbreeding *L. subnudus*^{wt} (SWT) with *L. edodes*^{wt} (EWT)

SWT: *L. subnudus* wild type ; SMT-060: *L. subnudus* mutant type obtained by exposure to UV-light for 60mins SMT-120: *L. subnudus* mutant type obtained by exposure to UV-light for 120 mins; *SMT-135: L. subnudus* mutant type obtained by exposure to UV-light for 135 mins

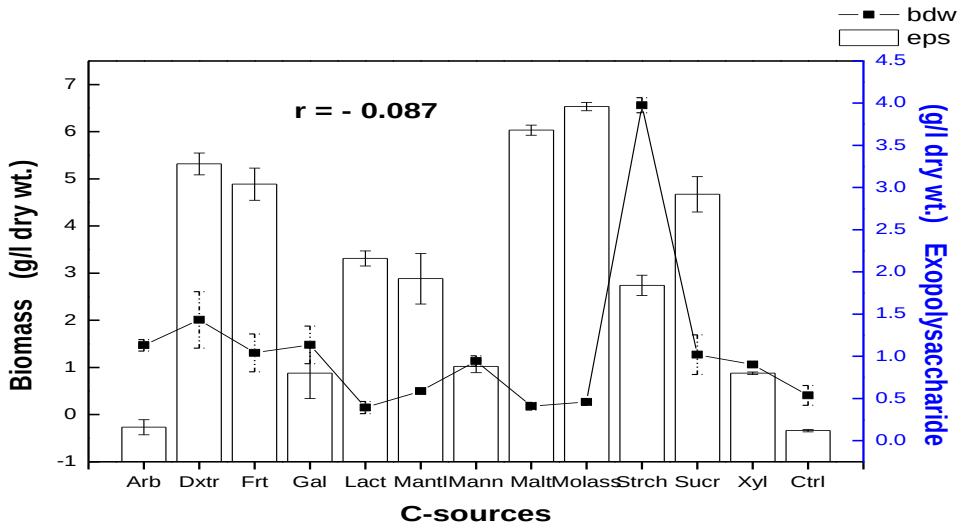


Fig. 1 Effect of different carbon sources on biomass and exopolysaccharide production in submerge culture of *L. subnudus*[wt]

Data are means of three replicates; Error bar represents standard error of means of observed values; r = Pearson correlation coefficient
Arb: arabinose; Dxtr: dextrose; Frt: fructose; Gal: galactose; Lact: lactose; Mantl: mannitol; Mann: mannose; Mol: molasses; Strch: starch; Sucr: sucrose; Xyl: xylose; Ctrl: control.

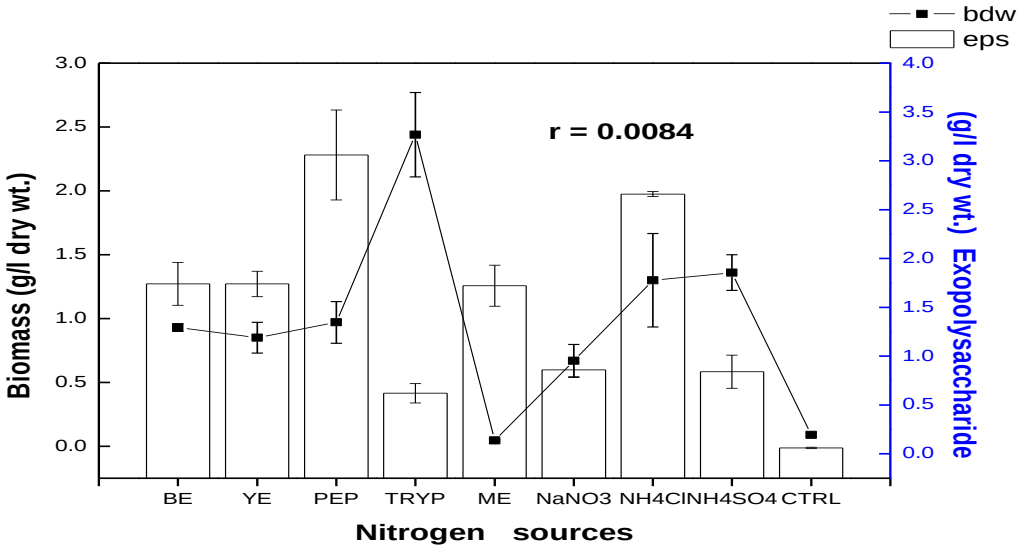


Fig. 2 Effect of different nitrogen sources on biomass and exopolysaccharide production in submerge culture of L. subnudus[wt]

Data are means of three replicates; Error bar represents standard error of means of observed values; r = Pearson correlation coefficient BE: beef extract; YE: yeast extract; PEP: peptone; TRYP: tryptone; ME: meat extract; CTRL: control.

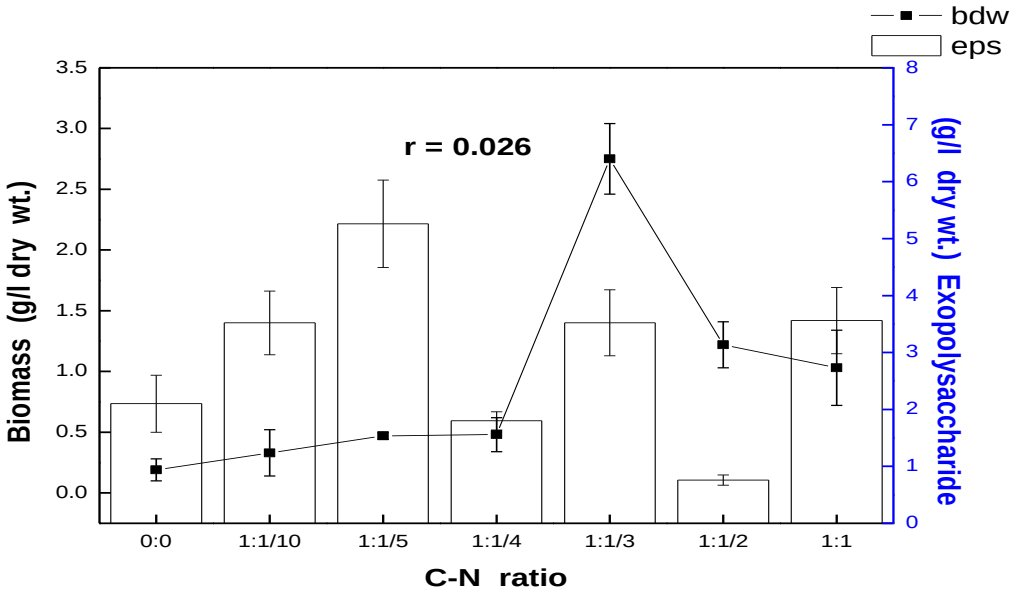


Fig. 3 Effects of carbon-nitrogen ratio on biomass and exopolysaccharides production in submerge culture of L. subnudus[wt]

Data are means of three replicates; Error bar represents standard error of means of observed values; r = Pearson correlation coefficient

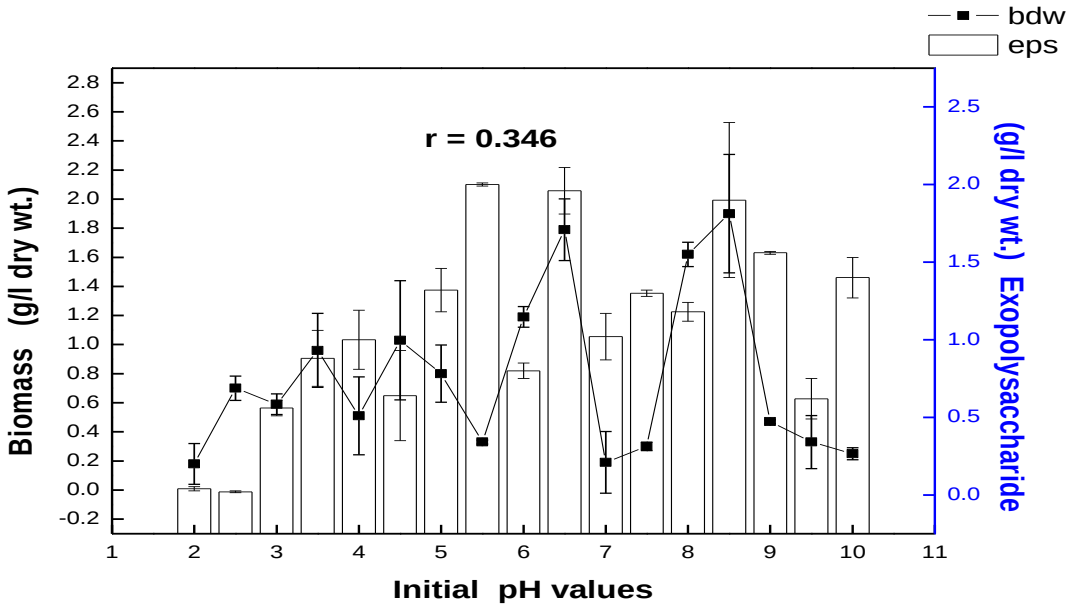


Fig. 4 Effect of initial pH on biomass and exopolysaccharide production in submerge culture of *L. subnudus*[wt]

Data are means of three replicates; Error bar represents standard error of means of observed values; r = Pearson correlation coefficient

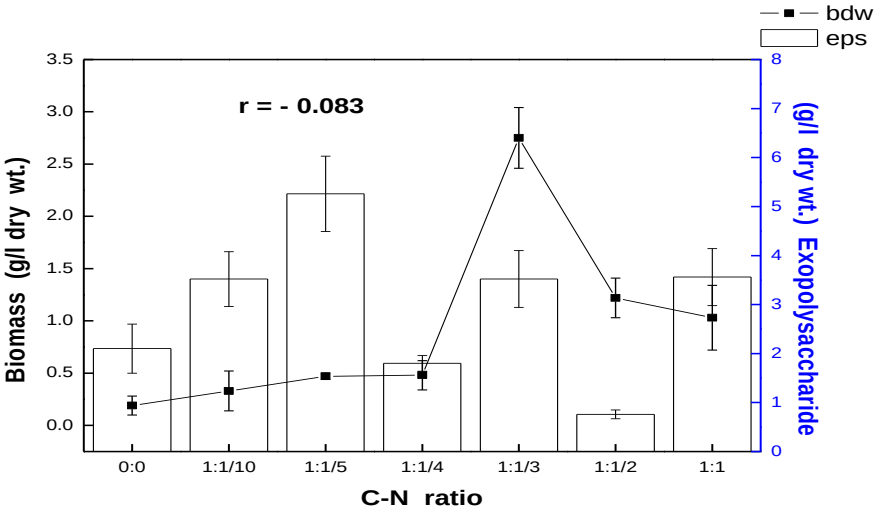


Fig. 5 Effect of seed culture volume on biomass and exopolysaccharide production in submerge culture of *L. subnudus*^{wt}

Data are means of three replicates; Error bar represents standard error of means of observed values; r = Pearson correlation coefficient