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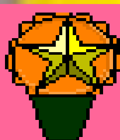

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Full Length Research Paper

Characterization of microbial degradation of oxytetracycline in river water and sediment using reversed phase high performance liquid chromatography

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The fate of oxytetracycline (OTC) in river water and sediment and control experiments was investigated. A high-performance liquid chromatography (HPLC) separation and identification method was used to separate, identify and quantify OTC and its major degradation products. Minimum degradation of OTC was observed in control experiments. Non microbial degradation observed up to day 26 contributed slightly above 20% of the degradation in exposed distilled water experiment. Increase in degradation of OTC after day 26 for both the covered and exposed distilled water experiments were attributed to microbial degradation due to contamination through the openings left in the set ups. Microbial degradation was observed in the river water and sediment experiment and two major degradation products were identified: 4-epi-oxytetracycline (4-epi-OTC) and β-apo-oxytetracycline (β-apo-OTC). β-apo-OTC was found to be the most stable degradation product as compared to the other main degradation products 4-epi-oxytetracycline (4-epi-OTC) and α-apo-oxytetracycline, (α-apo-OTC). The present results have shown that microbial degradation plays a major role in the removal of OTC in natural environments.

Key words: Oxytetracycline, microbial degradation, rephrased phase HPLC, river water and sediment.

INTRODUCTION

Contamination of aquatic environment with antibacterial agents has been a subject of discussion by many authors (Winckler and Grafe, 2001; Zhou et al., 2011, 2013). Tetracycline antibacterials are the widely applied antibiotics worldwide. More than 2500 tonnes are used annually in Europe and 21000 tonnes in China (Zhou et al., 2013) with oxytetracycline being the most used antibiotic. In Africa, tetracycline antibacterials are the widely applied antibiotics because they can be used to treat diseases caused by both Gram positive and Gram negative bacteria. They are also cheap. Oxytetracycline (OTC) has been detected widely in surface waters and soil with concentration reaching up to 2 mg L^{-1} (Ooishi and Tosa, 2010). Recently, Zhou et al. (2013) and Yang et al. (2011)

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monitored different types of environmental samples and detected frequently tetracyclines than any other antibiotics. Oxytetracycline has been reported to degrade abiotically in solution therefore many studies that have been done so far concentrated on photodegradation and hydrolysis of oxytetracycline in buffered and humic acid added distilled water or filtered surface waters (Xuan et al., 2010; Harring-SØrensen et al., 2003). Photodegradation products either in distilled water or filtered environmental waters that have been identified include, 4-epi oxytetracycline (4-epi-OTC), α-apo-oxytetracycline, (α-apo-OTC), β-apo-oxytetracycline (β-apo-OTC) (Xuan et al., 2010; Kuhne et al., 2001) and in addition 4-epi-N-desmethyl-OTC, N-desmethyl-OTC, N-didesmethyl-OTC and 4-epi- N-didesmethyl-OTC (Halling-SØrensen et al., 2003). Insights emanating from these studies are now being applied to treat drinking water of pharmaceuticals. Effectiveness of UV, ozone and other advanced oxidation techniques to degrade tetracyclines have been reported in literature (Wu and Chen, 2010; Chung et al., 2009). These techniques have some limitations because they can only be applied to treat clear water and they are expensive. If effluents from farms, hospitals and municipal sewage works are to be rendered free of pharmaceuticals more cost effective strategies still need to be sort. Pharmaceuticals have been assumed not to degrade microbiologically because they are bacteriostatic (Halling-SØrensen et al., 2003). Recent development has shown that micro-organisms in the environment have the potential to degrade tetracyclines and many other antibiotic types (Maki et al., 2006). A review of literature show limited information on the microbiological degradation of tetracyclines in the aquatic environment where photolysis is expected to be hampered by presence of radiation attenuating materials (Xuan et al., 2010). Therefore this study was aimed at studying microbial degradation of oxytetracycline in river water and sediment. Microcosm experiments were setup so as to resemble the real aquatic environment. High performance liquid chromatography has been the widely chosen method for analysis of environmental samples (Halling-SØrensen et al., 2003; Xuan et al., 2010). This is because there is a need to separate the parent compound from its degradation products. A previously reported high-performance liquid chromatography (HPLC) method (Xuan et al., 2010; Chinese pharmacopeia, 2005) was modified and used to separate oxytetracycline and its degradation products from river water and sediment.

MATERIALS AND METHODS

Chemicals

Oxytetracycline hydrochloride M_w, 496.9 g (OTC), 4-epi-oxytetracycline M_w, 460, 4 g (4-apo-OTC) α-apo-oxytetracycline M_w, 442.4 g (α-apo-OTC), β-apo-oxytetracycline M_w, 442.4 g (β-apo-OTC) were purchased from Sigma Aldrich, Darmstadt, Germany. All were of 95-98% purity. Methanol (HPLC grade), primary and secondary amine sorbent material (57738-U-SUPELCO supelclean PSA), acetonitrile (HPLC grade) and nylon disposable filter units (MILLPORE 0.45 µm) were also obtained from Sigma Aldrich. Oxalic acid, orthophosphoric acid, nitric acid, sodium hydrogen phosphate, citric acid and disodium ethylenediamine tetraacetate (Na_2EDTA) were of analytical grade and were obtained from SKYLABS, Gauteng, South Africa. River water (80 L) and sediment (2 kg) was collected from Wayerera River, Bindura, Zimbabwe (19° 19' 52'' South, 42° 21' 52'' East).

High-performance liquid chromatography (HPLC) system

OTC and its metabolites were analyzed according to a procedure reported in the Chinese pharmacopeia (2005) and by Xuan et al. (2010). A Varian HPLC UV Prostar 325 equipped with a Rodyne manual injector, a 20 mL loop and a Prostar 325 UV detector was used to analyze OTC and its metabolites. The detector was controlled remotely by the Varian Star/ Galaxie Chromatography Workstation software version 6. All HPLC separations were carried out using C18 (Varian Microsorb MV 1005 packed columns (250 × 4.6 mm id, 5 µm SPELCO). A mixture of methanol, acetonitrile and 0.01 M aqueous oxalic acid in the ratio of 1:1.5:7.5, pH 3.0 offered the best resolution of parent compound and its metabolites Figure 1. The flow rate was maintained at 1.0 $ml min^{-1}$ in the isocratic mode, at ambient temperature. A sonicator was used to mix and remove air bubbles from the mobile phase prior to HPLC analysis. The detector was set at 360 nm (the absorbance at maximum wavelength was determined using a UV-Vis instrument, GENESYS 10S UV-Vis v4.003 2L9Q129001, Thermofisher). Sample injection volume was 10 µL.

Preparation of standard samples

Stock solutions of OTC, 4-apo-OTC, α-apo-OTC and β-apo-OTC of concentration 1×10^{-3} g mL^{-1} were prepared in methanol and kept in refrigerator in amber bottles. Duplicate calibration curves were made by diluting the stock solutions in the range 0.01-1 µg mL^{-1}.

Microbial degradation experiments

Microcosm experiments were set up following a previous method described by Zaranyika and Nyoni (2013) with some modifications. Volumes of 1 × 80 L of river and 2 × 80 L each of distilled water were added into separate 80 L white plastic tanks (Mega Pak Zimbabwe (Pvt), Harare), and levels marked. Two kilograms of sediment was added in the vessel with river water. Control experiments consisting of distilled water and heat sterilized river water and sediment were also set as follows: one vessel containing distilled water was covered with aluminium foil to prevent light penetration, but making sure that air was not excluded. The other tank with distilled water was left exposed to sunlight. The last tank consisted of heat sterilized river water and sediment. Heat was applied cautiously so that only microbes would be destroyed without destroying humus. The tanks were spiked with standard oxytetracycline dissolved in methanol so as to obtain a final concentration of 1.2 µg mL^{-1}. The contents were then mixed thoroughly. The system was left to settle for 1 h and samples taken immediately thereafter. Containers were covered with perforated transparent polythene and left outside in a safe place near the Bindura University laboratory. Thereafter samples were collected periodically for a period of 90 days, each time compensating for evaporation by adding distilled water 24 h prior to sampling. Sediment samples were collected from the bottom of tanks with minimum agitation using a stainless scoop. The temperature and

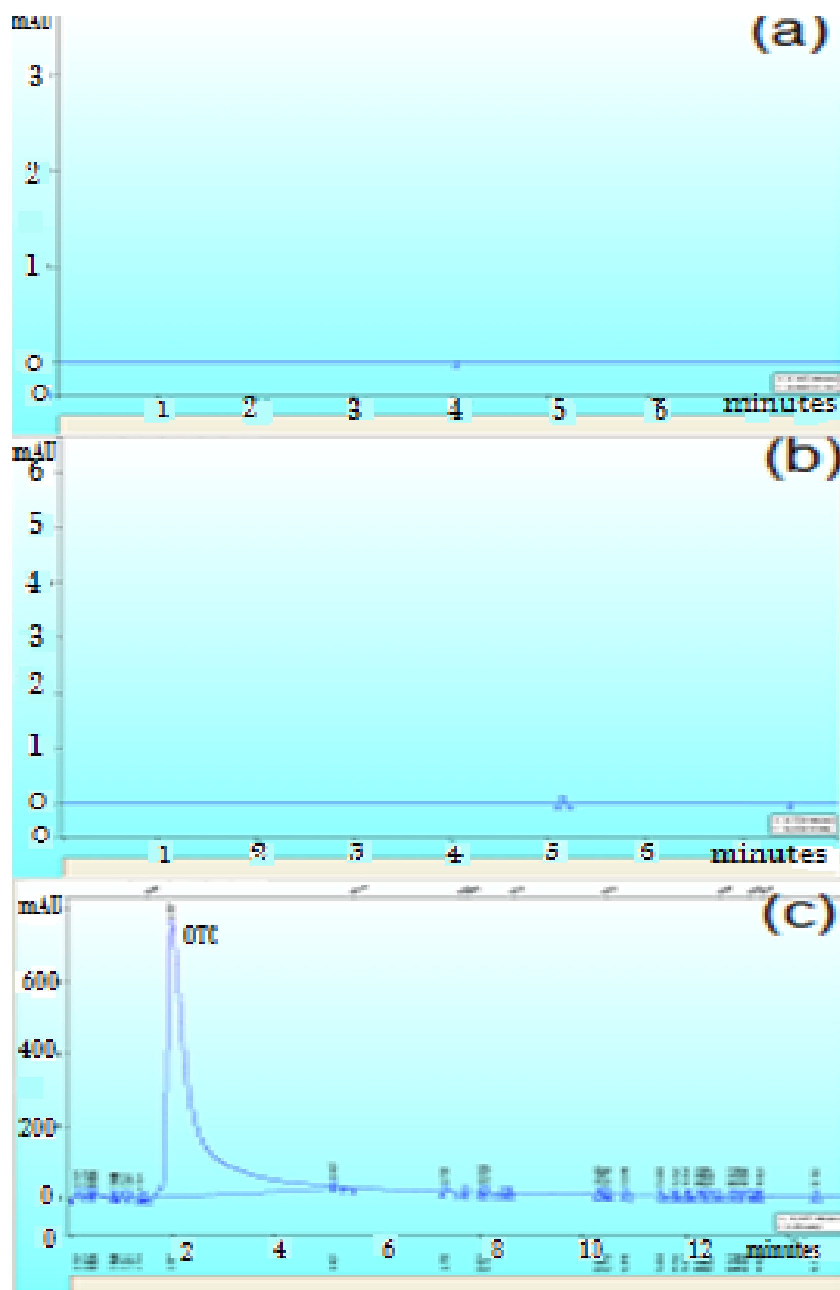

Figure 1. Selected chromatograms for river water, (a) blank river water sample, (b) blank sediment sample, (c) chromatogram for day 34, (d) 64, (e) and (f) 90.

pH of each tank were recorded before each sampling. The following data were obtained: temperature of 27 ± 5°C; pH, 7.2 ± 0.4 (river water); temperature 28 ± 3°C and 5.3 ± 0.8 (distilled water). Once collected, all samples were stored in a freezer in plastic bottles with screw caps until required for analysis.

Sample extraction, clean up and concentration

OTC was extracted from water and sediment samples using ultrasonic assisted dispersive solid phase extraction (UA-DSPE). Dispersive solid phase extraction was previously used to extract pharmaceuticals from food samples (Cruz-Vera et al., 2011).

Water samples

Water samples were analyzed in triplicate. One hundred milliliters of water samples were centrifuged at 3000 rpm. The supernatant was collected and vigorously shaken with 10 mL of acetonitrile in a separating funnel. Five milliliters of 0.1 M Na_2EDTA, and 10 mL of McIlvaine buffer (pH 4) were also added to chelate any metals present. Magnesium sulphate and sodium chloride 0.5 g each were then added to displace the extraction equilibrium towards the

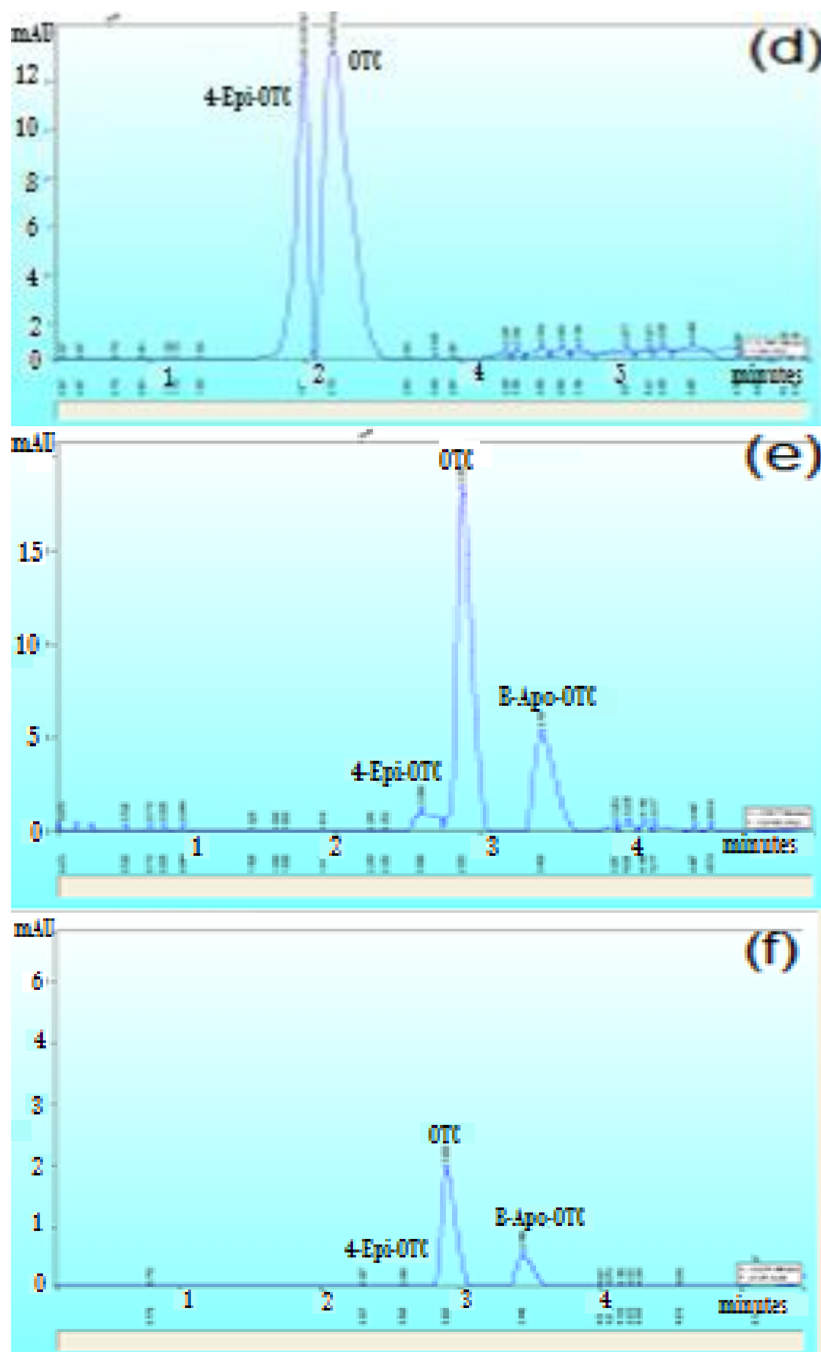

Figure 1. Contd

organic phase. The contents were centrifuged at 3000 rpm for 10 min and the organic supernatants were transferred to a conical flask followed by addition of 40 mg of primary secondary amine sorbent material (57738-U-SUPELCO Supelclean PSA) to remove interferences such as humic acid (Cruz-Vera et al., 2011). The analyte of interest remained in the organic phase. The mixture was ultrasonicated for 15 min and centrifuged at 3000 rpm for 10 min. The supernatants were collected and evaporated to almost dryness under vacuum and then redissolved in 500 µL of methanol. The contents were filtered through a 0.45 µm glass Millipore filters to remove any particulate matter and then placed into amber vials and stored in a fridge until HPLC-UV analysis (Zhou et al., 2011).

Sediment samples

Two grams of sediment samples were extracted after removing excess water by centrifugation. Ten milliliters of McIlvaine buffer (pH 4) were added into each glass tube and mixed for 1 min, then centrifuged at 3000 rpm for 10 min. The supernatants from each

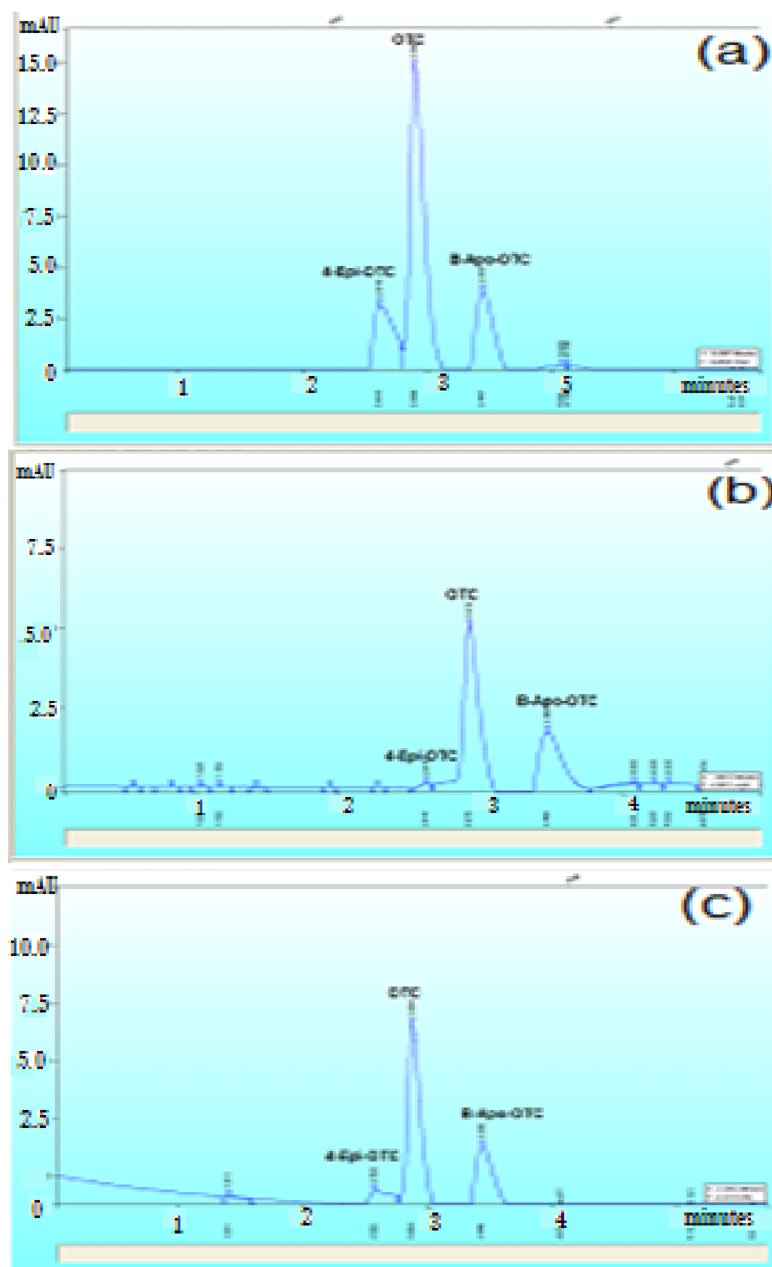

Figure 2. Sediment sample selected chromatograms for days: (a) 26, (b) 34, (c) 64, (d) 72, (e) 90.

tube were placed into 250 mL flasks. The extraction process was repeated twice and the supernatants from the two extractions were combined and diluted to 100 mL with ultrapure water and vigorously shaken with 10 mL of acetonitrile in a separating funnel. Five milliliters of 0.1 M Na_2EDTA, and 10 mL of McIlvaine buffer (pH 4) were also added to chelate any remaining metals, after which the extraction was carried out as described above for water samples.

RESULTS AND DISCUSSION

Degradation products

In the present study degradation products peaks (Figures 1 and 2) t_R(4-apo-OTC) = 2.38 ± 0.34 min and (β-apo-OTC) = 3.26 ± 0.32 min were only visible when the concentration of the parent peak had reduced to 0.06 µg/mL such that they were not visible in the covered and exposed distilled water experiments since the concentration did not drop to such levels. Degradation product α-apo-OTC) was not detected. The reasons might be that it was in very small quantities or it degraded quickly as it was formed. In an almost similar study that was conducted by Halling-SØrensen et al. (2003) α-apo-OTC was found to degrade faster in light than its counterpart epimer β-apo-OTC. Degradation products

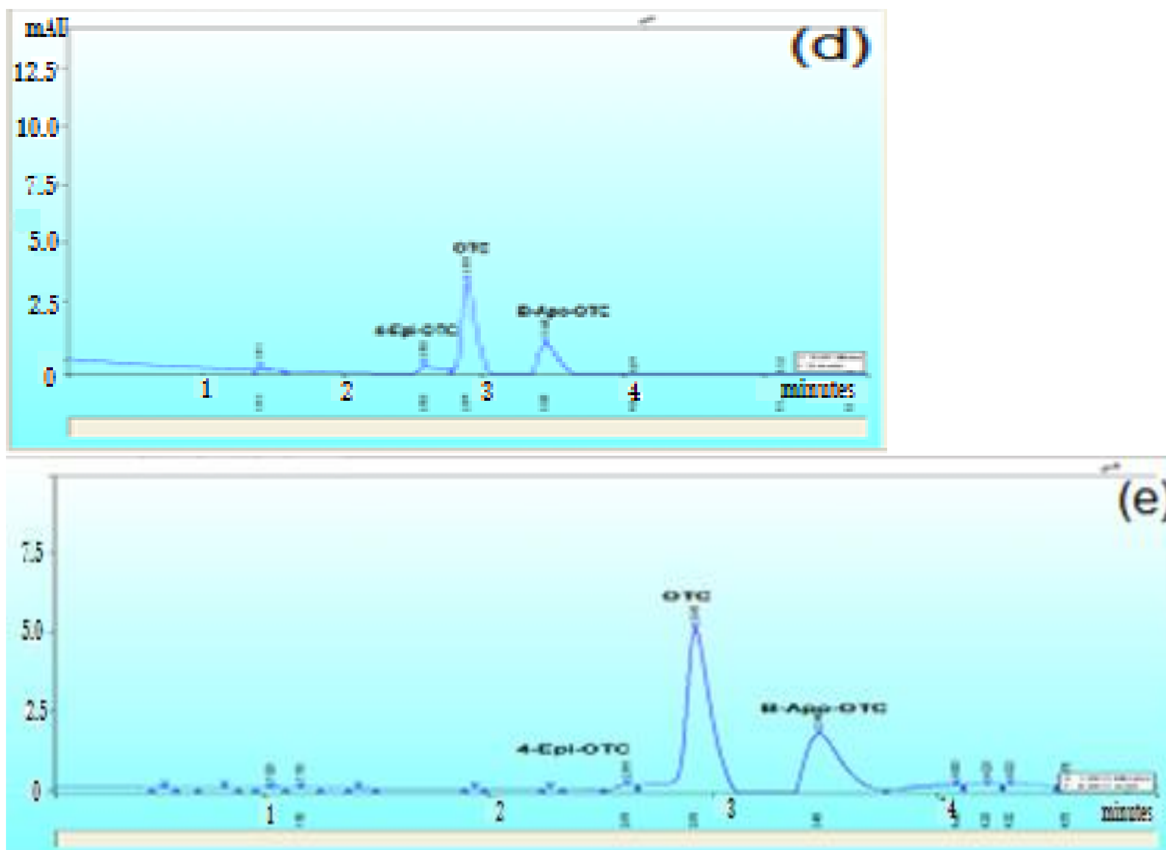

Figure 2. Contd

such as 4-epi-N-desmethyl-OTC, N-desmethyl-OTC, N-didesmethyl-OTC and 4-epi- N-didesmethyl-OTC that were reported in Halling-SØrensen et al. (2003) study was not detected in this study. The major reason might be the different spiking concentrations that were applied. For this study very low concentrations were chosen so as to mirror as close as possible concentrations in the real environment while Halling-SØrensen and coworkers used a higher spiking level that ensured detection of very small fractions of degradation products as compared to parent compound.

Degradation of OTC in microcosm and control experiments

Degradation experiments were performed over 90 days and followed the transformation of OTC in distilled water both exposed to light and in the dark, river water and sediment sterilized and unsterilized. Figures 3 and 4 show the concentration of OTC and its metabolites respectively versus time over the period of degradation study. Slow degradation of OTC (Figure 3a) was initially observed in the covered distilled water experiment however slight increase in degradation was observed from day 26. This was attributed to microbial degradation since the experiment consisted of openings to allow exchange with the environment. Furthermore no significant difference was observed between the results obtained from the covered distilled water set up and sterilized river water and sediment showing that no other transformation process other than hydrolysis was significant. Tetracyclines have been reported to degrade abiotically by Fenton reactions involving Fe^{2+} and by oxidation in the presence of metal oxides (Chen and Huang, 2011). Also traces of algae were observed at the end of the experiment. Xuan et al. (2010) performed similar experiments with modified distilled water in the dark and also observed slow degradation of OTC. Similar profiles were observed by Doi and Stoskopf (2000) in their study of the degradation of OTC in deionized water however they did not discuss the issue. Much faster degradation as compared to the covered distilled water experiment was observed in the exposed distilled water setup Figure 3(b). This can be attributed to photo-degradation. OTC has been found to degrade readily in distilled water under UV light irradiation (Doi and Stoskopf, 2000). A change in degradation speed was also observed from day 26 and this was attributed to microbial degradation. Contamination by micro- organisms in the atmosphere

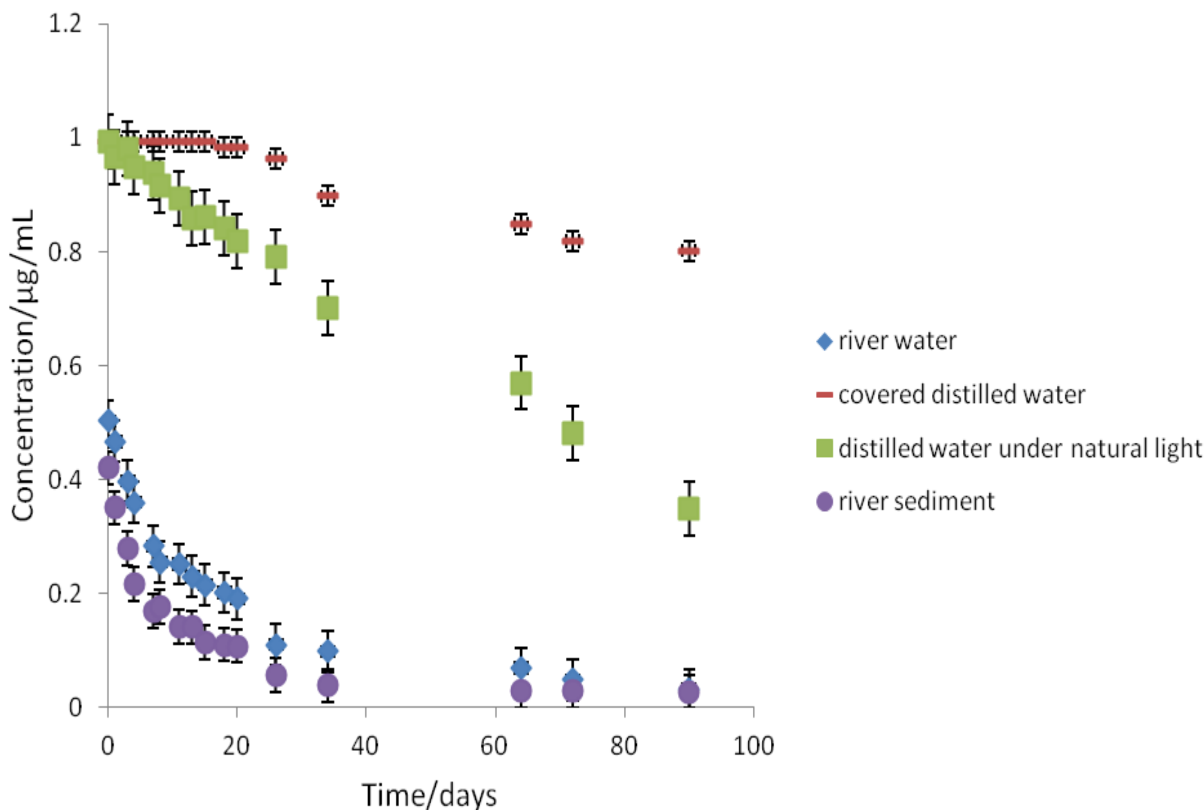

Figure 3. Concentration of oxytetracycline (OTC) in river water, covered distilled water and distilled water exposed to sunlight, river sediment.

may have occurred through the transparent plastic perforations. Abiotic degradation under natural conditions contributed to slightly above 20% degradation in the exposed distilled water experi-ment over the 90 day period showing that OTC can persist under natural conditions if microbes that degrade the antibiotic are absent. In filtered and autoclaved soil, interstitial water Halling-SØrensen and coworkers observed a general stability of OTC molecules with half-lives up to 270 days.

Significant degradation was observed in the river water and sediment Figure 3(c) and (d) and this can be attributed to microbial degradation. Photodegradation was not expected to be significant in the study by looking at results from the control experiments. Photodegradation is only significant in shallow clear water and is expected to be hampered by presence of soil and organic particulate matter in the naturally relevant conditions as employed in this study (López-Peñalver et al., 2010). Traces of algae were also observed in the microcosm experiments. It has been reported in previous studies that algae can degrade organic molecules such as tetracyclines by using them as a source of carbon by releasing enzymes into the solution (Migliore et al., 2012).

Figure 4 a-d shows the disappearance of the main degradation products in river water and sediment spiked with OTC. The Figures depict β-apo-OTC to be the dominant degradation product whereas in similar studies α-apo-OTC was the dominant product (Loke et al. (2003). Halling-SØrensen et al. (2003) also observed β-apo-OTC to be the predominant degradation product. 4-epi-OTC reached the highest concentration in this study as compared to the other degradation products. Formation of 4-epi-OTC reached a mole fraction of 0.2-0.3 to the parent compound at day 64 in river water Figure 1 (d). These results are almost in agreement with what was reported by Halling-SØrensen et al. (2003). In their study more than 60% of OTC was converted to 4-epi-OTC after 100 days. Conversion from OTC to β-apo-OTC Figure 5 involves loss of a water molecule.

The degradation therefore most likely involves dehydrolase enzymes released from micro-organisms and algae (Nnenna et al., 2011). These enzymes can be extracted, incubated, optimized and applied in the remediation of contaminated sites and to treat effluents from farms, pharmaceutical industry, hospital and municipal effluents before they can be released into the environment. Very few studies have been devoted to this regard. Meyers and Smith (1962) investigated the application of *Xylaria digitata*, a fungi to degrade tetracycline antibiotics and found out that they were effective. Other investigations involving the use of microbial degradation are reported by Maki et al. (2006).

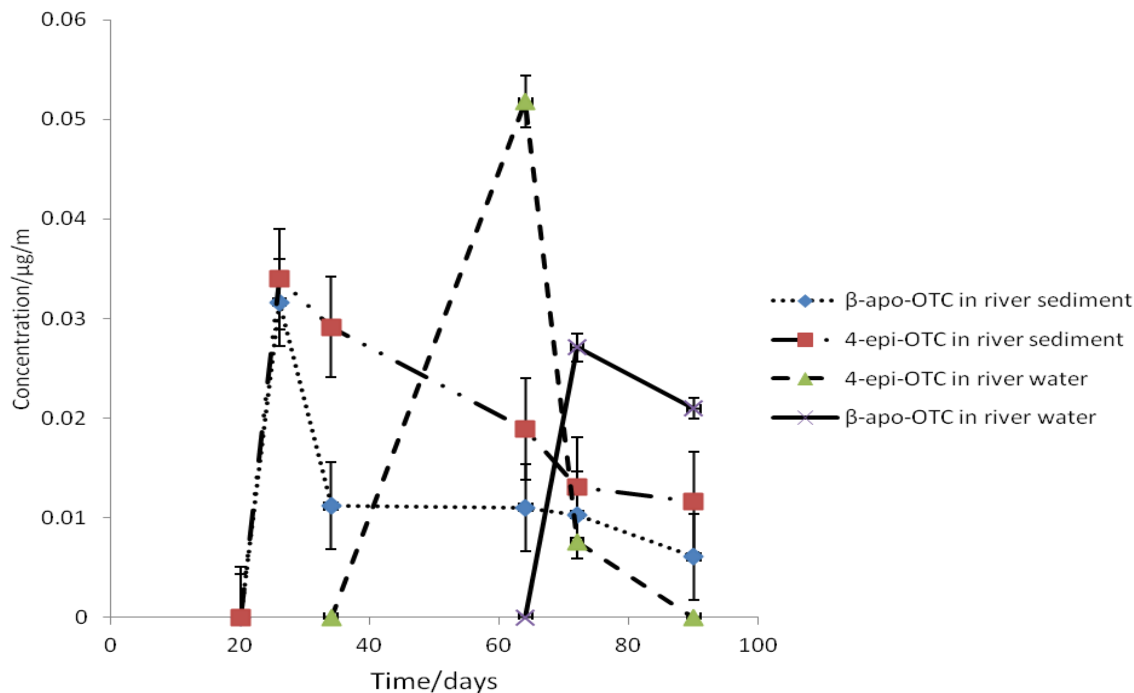

Figure 4. Concentration of, 4-epi-oxytetracycline (OTC), β-apo- oxytetracycline (OTC) in river sediment and water.

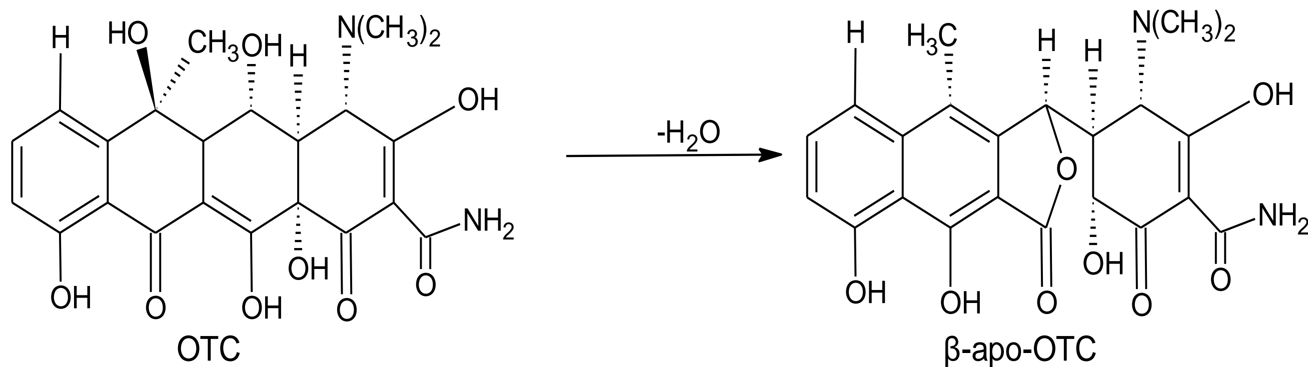

Figure 5. Hydrolysis of oxytetracycline (OTC) to β-apo-oxytetracycline (OTC).

and Wen et al. (2009).

Conclusion

Results of the present study show that microbial degradation plays an important role in the removal of OTC in the aquatic environment. All degradation products were present in trace levels. The study found out that in river water and sediment β-apo-OTC is the most stable degradation product. Microbial degradation of OTC to 4-epi-OTC reached a higher mole ratio, 0.2:0.3 as compared to the parent compound as other compounds.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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African Journal of Biotechnology

Full Length Research Paper

Comparison of fatty acid profile of wild and farm reared freshwater prawn *Macrobrachium rosenbergii* (De Man) brooders for broodstock diet formulation

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Fatty acid profiles of midgut gland (MG), ovary and eggs of *Macrobrachium rosenbergii* of wild and farm reared brooders indicate a significant variation of their components during the sexual maturation and spawning. In both groups, major fatty acids found in the chosen tissues were 14:0, 16:0, 18:0, 18:1n-9, 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3 and 22:6n-3. Of these, saturated fatty acids dominate over the mono-unsaturated (MUFA), polyunsaturated (PUFA) and highly unsaturated fatty acids (HUFA). Though all the four groups of fatty acids are found in both groups except n-3 HUFA (P>0.05) other categories are found in higher wild brooders (P<0.05). There was a significant difference in total weight, total length and clutch weight (P<0.05) between farm and wild brooders, but the gonadosomatic index (GSI) and midgut gland somatic index (MSI) did not vary significantly (P>0.05).

Key words: Fatty acids profile, morphometry, *Macrobrachium rosenbergii*, wild and farm brooders.

INTRODUCTION

A thorough knowledge on the physiology, metabolism and biochemistry of commercially important species during maturation is essential for a complete understanding of its reproductive processes for hatchery operation (Mourente et al., 1994); indeed reproductive control of a cultured species is importance only next to its consumer demand (Bardach et al., 1972). Reproduction in crustacean entails maternal mobilization, biosynthesis and bioaccumulation of materials for export as self sufficient capsules; the unfertilized eggs (Harrison, 1990). The eggs are rich in yolk substances that are used as nutrient for embryonic development processes. Usually protein, one of the main components of yolk, plays an important role in both morphogenesis and energy supply in embryos (Holland, 1978; Luo et al., 2004). In decapod eggs, lipid content is relatively high and constitutes one of the major energy sources. Lipids play an important role in embryonic metabolism as they are the most important energy source and provide at least 60% of the total energy expended by the developing crustacean embryo; during the stages of

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embryonic development, lipids also serve as the components of biological membranes and pigments of compound eyes (Wehrtmann and Graeve, 1998).

In the early 1990s, commercial stocks of giant prawn have experienced productivity decline due to inbreeding depression; this is believed to have resulted from brood stock sourced from grow-out ponds (New, 2000). The fecundity and brood quality of wild *M. rosenbergii* are far better than the farm reared females (Wilder et al., 1999). But wild stocks of *M. rosenbergii* have also declined in recent years, as a result of over-harvesting, habitat loss and increased pollution; this is further compounded by the seasonal availability of wild brooders (Hien et al., 1998). Decline in quality and quantity of wild population has been reported in Bangladesh, India, Indonesia, Malaysia, the Philippines and Thailand (New et al., 2000). Hence *M. rosenbergii* hatcheries are constrained to depend on brooders procured from farms (Hien et al. 1998).

Understanding the interaction between nutrition and reproduction, quantification of the nutrient requirement is essential to promote successful maturation and spawning which in turn enable year round hatchery seed production (Harrison, 1997). In this regard, fatty acids of n-6 and n-3 series play a vital role in the reproductive performance of crustaceans. Hence a through evaluation of fatty acid profile during maturation and spawning is essential for sustainable aquaculture.

In India, freshwater prawn culture industry declines in terms of growth and production due to poor seed quality, random selection of brooders by hatchery, improper management practices in culture and imbalanced feeds. In this study area also for the past one decade a vicious cycle of supply of brooders from farms to hatcheries and the resultant juveniles from hatcheries to farms (unpublished data). In this scenario, the present study aims to find out the variations in the chosen morphometry and biochemical profiles of brooders collected from two different farms are the main suppliers for hatcheries with the wild brooders to find out the differences in brood quality between wild and farm reared *M. rosenbergii* as a measure of brood quality assessment.

MATERIALS AND METHODS

Brooder collection

M. rosenbergii females captured from Vembanad lake (Lat 9º 28' & 10º 10' N and long 76º 13' & 31' E) Arookkutty Tool, Cochin, Kerala, India were transported to the laboratory in polyethylene bag filled with aerated freshwater with least disturbance. Farm reared brooders purchased from Kottaipatinum (9º 57' N, 79º 30' E, Puddukotai District, Farm I) and Srikandapuram, (10º 59' N, 79º 35'E, Nagapatinum District, Farm II) Tamilnadu, India were also transported under above condition to the laboratory. The prawns were measured individually for wet weight (± 0.1 g) and total length (±0.1 cm) (from tip of rostrum to the end of the telson). The prawns were then sorted into two groups: a) females with ripe ovaries extending beneath the third rostral spine of the cephalothorax and b) incubating females with bright yellow colour newly spawned eggs (Pandian and Balasundaram, 1982; Chang and Shih, 1995). In the first group (n=3) individuals were sacrificed and tissues were sampled from midgut gland and ovary. The gonado-somatic index (GSI) and midgut gland somatic index (MSI) were calculated as the percentage weight of the gonad and midgut gland (MG) to the total body weight respectively. Clutch weight was determined separately after removing excess water by blotting with filter paper; care was taken to sample eggs of the same stage since there is a significant variation in biochemical parameters during different developmental stages of the egg (Cavalli et al., 2001). All the samples were stored at -70°C until further analysis.

Biochemical analysis

Total lipid (Folch et al., 1957) and fatty acid methyl esters (FAME) (Miller and Berger, 1985) content of midgut gland, ovary and eggs were quantified adopting standard analytical procedures. FAME was analyzed using a Hewlett-Packard 5890 gas liquid chromatograph equipped with Diethylene Glycol Succinate (DEGS) column and flame ionization detector (FID) using nitrogen as the carrier gas. The column temperature was 180°C and injection temperature was 200°C. The detector response was recorded. The FAME were identified and quantified by comparison of peak area and retention time of standard fatty acids.

Statistical analysis

The biometric variables such as total weight, total length, gonadosomatic index (GSI), midgut gland somatic index (MSI), clutch weight and selected tissue biochemical composition of *M. rosenbergii* brooders of wild and farms are analyzed through one-way analysis of variance (ANOVA). Subsequently the significant means are ranked through multiple mean comparison tests with LSD.

RESULTS

Morphometry

The biometric variables of *M. rosenbergii* females are summarized in Table 1. There were no significant differences found in GSI and MSI of the brooders between wild and farms but total wet weight, total length and clutch weight varied significantly ($P<0.05$) between wild and farm brooders.

Fatty acid profile

The fatty acid profile of midgut gland, ovary and eggs of brooders collected from wild and farms are compared. The MG of wild brooder have maximum total lipid (34.3%) content than the farm reared brooders (Table 2) and the difference is statistically significant ($P<0.05$). The total lipid content of farm I (29.5%) and farm II (28.5%) also differed significantly ($P<0.05$). Fatty acids found predominantly in all these organs are saturated fatty acids like myristic (14:0), palmitic (16:0), stearic (18:0) and monounsaturated fatty acid such as oleic (18:1n-9) acid.

Table 1. Total weight, total length, GSI, MSI and clutch weight of wild and farm brooders of *M. rosenbergii*. Superscript letters within rows indicate significant differences (P<0.05). Values are mean ± SD (n=10).

Variable	Wild	Farm I	Farm II
Total weight (g)	44.5±13.4^{b}	42.4±12.7^{ab}	40.3±12.0^{a}
Total length (cm)	17.4±2.9^{c}	15.5±2.5^{a}	16.8±2.7^{b}
GSI (%)	5.1±0.9	4.9±0.8	4.8±0.8
MSI (%)	4.0±0.6	3.8±0.6	3.9±0.5
Clutch weight(g)	4.1±0.8^{b}	3.1±0.6^{a}	3.2±0.1^{a}

Table 2. Selected principal fatty acid content (mg g^{-1} dry weight) of the MG of wild and farm broodersof *M. rosenbergii*. Each value is a mean ± S.D (n=3). Different superscripts within rows represent significant differences (P<0.05).

Fatty acid	Wild	Farm I	Farm II
Total lipids	34.3±2.4 ^{c}	28.5±1.9^{a}	29.5±2.1^{b}
14:0	38.04±2.3^{c}	11.61±1.2^{b}	5.50±0.68^{a}
16:0	32.03±2.2^{c}	5.53±0.61^{a}	7.96±0.95^{b}
18:0	28.83±1.7^{c}	5.89±0.64^{a}	6.44±0.77^{b}
18:1n-9	33.82±2.4^{c}	4.54±0.59^{a}	6.76±0.87^{b}
18:2n-6	28.73±2.0^{c}	10.71±1.07^{a}	11.89±1.30^{b}
18:3n-3	7.46±0.52^{c}	4.47±0.63^{a}	5.57±0.72^{b}
20:4n-6	5.51±0.44^{c}	2.04±0.76^{a}	3.29±0.46^{b}
20:5n-3	5.02±0.40^{a}	5.82±0.75^{a}	6.96±0.90^{b}
22:6n-3	4.59±0.41^{a}	5.31±0.48^{a}	6.59±0.72^{b}
Σ Saturates*	98.89±4.94^{b}	23.04±2.3^{a}	20.89±2.29^{a}
Σ Mono-unsaturated**	33.82±2.4^{c}	4.54±0.59^{a}	6.76±0.87^{b}
Σ n-6 PUFA	34.24±2.74^{c}	12.75±1.40^{a}	15.19±1.36^{b}
Σ n-3 HUFA	17.06±1.36^{a}	15.59±1.24^{a}	19.12±1.72^{b}
DHA/EPA ratio	0.913	0.912	0.947
n-6/n-3 ratio	2.01	0.817	0.794

*Saturates: 14:0; 16:0; 18:0. **Monounsaturates: 18:1n-9, n-6 PUFA: 18:2n-6; 20:4n-6. n-3 HUFA: 18:3n-3; 20:5n-3; 22:6n-6.

The n-6 polyunsaturated fatty acids (PUFA) such as linoleic (18:2n-6) and arachidonic acid (20:4n-6) and n-3 highly unsaturated fatty acids such as eicosapentaenoic (20:5n-3) and docosahexaenoic acid (22:6n-3) are present at an intermediate levels in all the tissues studied.

The comparison of fatty acids of MG indicate that the wild brooders (98.8 mg g^{-1}) have maximum saturated fatty acids which is significantly (P<0.01) different from farm reared brooders. But there was no such significant difference (P>0.05) between farm I (23.0 mg g^{-1}) and farm II (20.8 mg g^{-1}) brooders. There was significant difference in the amount of monounsaturated fatty acid (18:1n-9) (P<0.01) in the wild brooders (33.8 mg g^{-1}) when compared with Farm II (6.8 mg g^{-1}) and Farm I (4.5 mg g^{-1}) brooders. The content of n-6 PUFA were also significantly higher (P<0.05) in wild (34.2 mg g^{-1}) than the farm reared brooders. Interestingly in MG, the n-3 HUFA level showed a significant variation between farm and wild brooders.

The total lipid and fatty acid profile of ovary of wild and farm reared brooders are presented in Table 3. The total ovarian lipid content of wild brooders was 37.4%, which is differed significantly (P<0.05) when compared to farm I (33.5%) and Farm II (33.7%). Though there is a variation in farm and wild among the farm reared brooders there is no such difference. Saturated fatty acids are found higher in the ovary of wild brooder (52.8 mg g^{-1}) than the farm reared brooders (P<0.05). Monounsaturated fatty acids 18:1n-9 was found rich in wild (35.3 mg g^{-1}; P<0.01) followed by farm II (7.6 mg g^{-1}) and farm I (6.3 mg g^{-1}) (P<0.05) reared brooders. The n-6 series of polyunsaturated fatty acids of wild brooder (35.5 mg g^{-1}) was significantly higher (P<0.05) than Farm I (16.4 mg g^{-1}) and Farm II brooders (15.4 mg g^{-1}). There was no significant difference in n-3HUFA level among brooders

Table 3. Selected principal fatty acid content (mg g^{-1} dry weight) of the ovary of wild and farm brooders of *M. rosenbergii*. Each value is a mean ± S.D (n=3). Different superscripts within rows represent significant differences (P<0.05).

Fatty acid	Wild	Farm I	Farm II
Total lipids	37.4±2.99^{b}	33.5±3.02^{a}	33.7±3.03^{a}
14:0	14.59±1.60^{c}	4.53±0.41^{a}	5.97±0.55^{b}
16:0	23.89±2.62^{b}	9.76±0.87^{a}	9.68±0.77^{a}
18:0	14.41±1.58^{c}	9.93±0.89^{b}	8.03±0.72^{a}
18:1 n-9	35.39±3.89^{c}	6.39±0.61^{a}	7.67±0.69^{b}
18:2 n-6	30.57±3.36^{c}	14.45±1.58^{b}	13.03±1.43^{a}
18:3 n-3	4.53±0.45^{a}	6.26±0.55^{b}	5.59±0.63^{ab}
20:4 n-6	4.95±0.44^{b}	1.98±0.39^{a}	2.43±0.52^{a}
20:5 n-3	6.59±0.59^{a}	6.98±0.62^{a}	7.78±0.70^{b}
22:6 n-3	6.77±0.61^{a}	5.28±0.61^{a}	6.05±0.54^{a}
Σ Saturates	52.89±5.82^{b}	24.23±2.66^{a}	23.68±2.84^{a}
Σ Mono-unsaturated	35.39±3.89^{c}	6.39±0.61^{a}	7.67±0.69^{b}
Σ n-6 PUFA	35.51±3.91^{b}	16.43±1.81^{a}	15.46±1.70^{a}
Σ n-3 HUFA	17.89±1.96^{a}	19.53±2.14^{a}	19.44±2.13^{a}
DHA/EPA ratio	1.050	0.848	0.788
n-6/n-3 ratio	2.053	0.846	0.804

Table 4. The selected principal fatty acid content (mg g^{-1} dry weight) of eggs of wild and farm brooders of *M. rosenbergii*. Each values is a mean ± S.D (n=3). Different superscripts within rows represent significant differences (P<0.05).

Fatty acid	Wild	Farm I	Farm II
Total lipids	34.7±3.81^{b}	33.4±4.00^{a}	33.6±4.03^{a}
14:0	10.58±1.37^{c}	7.19±0.64^{b}	3.53±0.63^{a}
16:0	14.66±1.61^{c}	4.98±0.44^{a}	6.95±0.62^{b}
18:0	13.09±1.44^{b}	6.68±0.59^{a}	5.55±0.75^{a}
18:1n-9	30.59±3.36^{b}	5.74±0.47^{a}	6.15±0.52^{a}
18:2n-6	36.33±3.99^{c}	13.09±1.57^{b}	9.79±0.87^{a}
18:3n-3	3.91±0.35^{a}	4.41±0.62^{ab}	5.16±0.46^{b}
20:4n-6	3.83±0.66^{b}	1.57±0.63^{a}	2.03±0.24^{a}
20:5n-3	5.36±0.63^{ab}	4.71±0.53^{a}	6.06±0.54^{b}
22:6n-3	4.66±0.73^{a}	4.83±0.53^{a}	5.11±0.45^{a}
Σ Saturates	38.33±4.98^{b}	18.85±2.26^{a}	16.54±2.12^{a}
Σ Mono-unsaturated	30.59±3.36^{b}	5.74±0.47^{a}	6.15±0.52^{a}
Σ n-6 PUFA	40.16±4.82^{c}	14.66±1.76^{b}	11.82±1.42^{a}
Σ n-3 HUFA	13.94±1.67^{a}	13.95±1.68^{a}	16.33±1.95^{a}
DHA/EPA ratio	0.848	1.026	0.833
n-6/n-3 ratio	3.05	1.081	0.723

of wild and farms (P>0.05). Total lipid and fatty acids of eggs in wild and farm brooders show a similar trend like ovary (Table 4).

DISCUSSION

In India, commercial farming of *M. rosenbergii* culture face productivity declined in recent years. Poor quality of seeds and low feed conversion efficiency affect the growing industry. Previous studies on reproductive performance explained inbreeding depression as a major cause which is as a result of brood stock sourced from the grow-out ponds rather than from the wild (New, 2000; Mather and Bruyn, 2003). In *Macrobrachium* culture, brooders are chosen for seed production not on the basis

of size, but on the availability and ready to spawn. This results in selection of smallest brooders that in turn lead to a substantial reduction of mean size across generations and loss of performance (New, 1995).

In the wild, *M. rosenbergii* female first mature at a size of 20-40 g; eggs obtained from these females are of good quality and their larvae show high percent of survival. However, females of hatchery origin, which are cultured as broodstock often, mature at a size of 7-10 g. Selection of such a precociously mature females for seed production results in eggs and larvae of poor quality; offspring of these females may mature even more precociously (Wilder et al., 1999).

Among the various factors govern to the broodstock quality the prime factors is the nutritional status of the brooder (Harrison, 1990). The total lipid content of the tissues of wild brooders is reflected by the habit of the organism, that is omnivorous food habit in a natural ecosystem where it has ready access to variety of organisms such as worms, insect larvae, small mollusks, aquatic insects, fish and other crustaceans (Ling, 1969). This enhances the constant growth under natural conditions and suggests the organism's ability to meet out the seasonal food shortage (Sahavacharin and Pongsuwan, 1974).

Studies in crustacean brooders, indicate that there is an accumulation of lipid content in ovary when compare to the eggs and midgut gland. Such an increased in lipid content suggests that the dietary lipids stored in the MG are transferred to the ovary for the maturation (Harrison, 1990; Cavalli et al., 1999). As indicated above, the nutrition status of the organism determines the accumulation of nutrient reserves. In this study also there is a significant difference in total lipid content of wild brooders when compare to the farm reared ones.

The fatty acid profiles of MG, ovary and eggs of wild and farm brooders in this study showed that the proportion of saturated and monounsaturated fatty acids was higher than that of n-6PUFA and n-3HUFA series. Previous studies also support the present findings in *M. rosenbergii* eggs (Tidwell et al., 1998; Cavalli et al., 2000), newly hatched larvae (Roustaian et al., 1999), Juveniles (Chanmugam et al., 1983) and midgut gland and ovary of mature females (Cavalli et al., 2000). The wild brooder possesses highest level of saturated and mono-unsaturated fatty acids than the farm brooders in all the organs. The highest level of saturated and mono-unsaturated fatty acids accumulation in the tissue shows these organs are the major energy source for embryonic (Clarke et al., 1990) and early larval development (Roustaian et al., 1999).

The comparison of n-6PUFA and n-3HUFA content in the tissues show much difference in wild and farm brooders. The n-6PUFA fatty acids are found higher in all the organs compare to n-3HUFA. For *Penaeids* n-3HUFA, particularly EPA and DHA are considered as essential fatty acids (Kanazawa et al., 1979a; 1979). The maturation performance and offspring quality of *Penaeids* are linked to dietary n-3HUFA level (Middleditch et al., 1980; Teshima et al., 1998; Teshima et al., 1989; Xu et al., 1994). In the case of freshwater prawn, n-6PUFA series of fatty acids predominate over n-3HUFA fatty acids (Chanmugam et al., 1983). The essential role of n-6PUFA especially linoleic acid (18:2n-6) has been demonstrated for juveniles (Reigh and Stickney, 1989; D'Abramo and Sheen, 1993).

Increase in the amount of ovarian linoleic acid during maturation, indicates its importance in metabolism or its response. This increase influences the fecundity of *M. rosenbergii* (Cavalli et al., 1999). In the present study n-6PUFA fatty acids were abundantly present in wild brooders compare to farms; this may be true for the wild caught brooders of Vembanad lake where they have recorded a high fecundity of 227,161 eggs per female with a total length of 258 mm and weight of 208 g; the smallest female measuring a total length of 158 mm and a total weight of 33.7 g had a clutch size of 30,666 eggs (Sureshkumar and Kurup, 1998). The small sized farm reared brooder measured a total length of 155 mm and weight of 42.4 g had a clutch size 34,020 (Balamurugan, 2006). This gives a strong support on the role of richness in n-6PUFA in the wild when compared to farm brooders. Moreover, the high level of n-6 fatty acids, in wild brooders is not surprising because of its natural diets (Chanmugam et al., 1983).

Hence the evaluation of broodstock prawns captured at different times and different habitat is essential for quality seed production. Variation between these individuals and their nutritional condition play a vital role on brood quality (Marsden et al., 1997). The n-6/n-3 ratio of the farm brooder is higher than the wild brooders is due to the diet available in their environment. Increase in n-3 HUFA series of farm reared brooders of *M. rosenbergii* is as a result of formulated grow out diet which are rich in animal proteins of marine origin that are rich source of n-3 series of HUFA. In wild, shrimp maturation and reproduction are greatly influenced by environmental factors (Bray and Lawrence, 1992) and the type of autothonous food which includes a variety of food items (Ling, 1969). Changes in the abundance and distribution of these food determine the preference of n-3 and n-6 series of fatty acids and perform the pattern of reproductive activity (Crocos and Coman, 1997).

The n-3 HUFA content of ovary and eggs of farm I and farm II brooders are more than in the wild brooders. For instance the amount of docosahexaenoic and eicosapentanoic acid are more and these acids play an important role in hatching, larval survival and structural component of cell membranes and formation of central nervous system in embryo (Cavalli et al., 1999). In *M. rosenbergii* bioconversion ability of EPA to DHA is lacking (Teshima et al., 1992). A well-balanced feed enriched by the required fatty acids will enhance the synthesis of EPA to DHA (D' Abramo and Sheen, 1993).

Comparatively low level of n-3 HUFA series especially docosahexaenoic acid in tissues of wild brooders would adversely affect the hatchability which is evident from the brood stock collected from the wild (unpublished data). Hence while selecting the brood stock from the wild, the hatcheries should take care of the nutritional level especially with reference to the eicosapentanoic and docosahexaenoic acid levels since they determine the hatchability and larval survival.

Conclusion

The present study clearly explained the importance of n-6 PUFA and n-3 HUFA content in the brooders for fecundity, hatchability and larval survival of *M. rosenbergii* for sustainable culture. The wild brooders lipid and fatty acids analysis show more amount of n-6 PUFA content which increased their fecundity and their low level of n-3 HUFA content reduced the hatchability and larval survival. In such a way that the farm reared brooders lipid and fatty acid analysis show less amount of n-6 PUFA and high level n-3 HUFA content reflects reduced fecundity and better larval survive supplied to culture. So, the present study strongly recommend that maturation diet fortified with ingredients should have a rich sources of n-6PUFA and n-3 HUFA for enhanced fecundity, hatchability and larval survival for sustainable *M. rosenbergii* culture.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Utilization of sweet potato starches and flours as composites with wheat flours in the preparation of confectioneries

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Freshly harvested roots of sweet potato variety TIS 87/0087 were processed into flour and starch using standard methods. Blends of Wheat and sweet potato composite flour was developed in the ratios 80:20, 70:30, 60:40 and 50:50. Also, blends of wheat and sweet potato starch were developed in the ratios 80:20, 70:30, 60:40 and 50:50. Whole sweet potato flour and starch were also included where 100% wheat flour was used as control or standard. Functional properties of wheat: sweet potato composite flour showed that water absorption capacity ranged from 2.0-2.5 g/ml, oil absorption capacity: 1.5-2.5 g/ml and bulk density: 0.68-0.82 g/ml. Gelatinization temperature ranged from 49.00-70.25°C. Wheat: sweet potato composite starch showed that water absorption capacity ranged from 1.5-2.0 g/ml, oil absorption capacity: 1.0-2.0 g/ml and bulk density: 0.74-0.78 g/ml. Gelatinization temperature ranged from 48.0-65.5°C. Sensory evaluation scores showed that up to 40% inclusion of sweet potato starch gave acceptable bread. Up to 40 and 50% inclusion of starch or flour to wheat gave acceptable cakes with desired colours. Also, 50% inclusion of starch or flour to wheat gave acceptable chinchin for colour and general acceptability.

Key words: Sweet potato, flour, starch, confectionery, functional properties, sensory evaluation.

INTRODUCTION

Sweet potato is an important food security crop that feeds millions of people in the developing world. The crop is popular among farmers with limited resources and produces more biomass and nutrient per hectar than any other food crop in the world (Parkash, 1994). Sweet potato as food product is a source of energy, proteins, pro-vitamin A (β-carotene), vitamin C and iron (Dufour et al., 1996). In Nigeria, the roots are cooked and prepared in limited number of ways; most commonly boiled. They are also cooked with beans and other foods, and sometimes fried as chips (Woolfe, 1992). Sweet potato roots possess a variety of chemical compound relevant to human health. About 80-90% of sweet potato dry matter is made up of carbohydrate consisting mainly of starch and sugar with

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lesser amount of pectin and hemi-cellulose and cellulose. Sweet potato also contains protein (0.46-2.93%), dietary fiber (0.49-471%), lipids (0.06-0.48%) and ash (0.31-1.06%). It also contains essential minerals such as zinc, phosphorus, magnesium, potassium and iron. Sweet potato is also an important source of pro-vitamin A, thiamine, riboflavin, niacin, ascorbic acid and many other functional compounds (Woolfe, 1992). Sweet potato tuber and leaves also contain anti-nutrients such as phytate, oxalate and tanins (Fleming, 1981; Udoessien and Ifon, 1990; Osagie, 1990). Sweeetpotato is now consumed mainly for its nutrients rather than for its energy (Jack et al., 1992). The crop has high Beta-carotene than any other root and tuber crops (Suda et al., 1999). Orange fleshed sweet potato is very rich in pro-vitamin A (Jack et al., 1992).

Sweet potato starch is isolated same way like other starchy roots except that the solution is kept alkaline (pH 8) using lime which help to dissolve the pigment (Akoroda and Egeonu, 2009). Unlike cassava and maize starches, sweet potato starch does not have high viscosity values on pasting and gelatinization temperature (Garcia, 1993). Sweet potato flour can serve as a source of energy and nutrients: carbohydrate, beta-carotene (pro-vitamin A), minerals and can add natural sweetness, colour flavour and dietary fiber to processed food products (Woolfe, 1992; Ulm, 1988).

Bread may be described as a fermented confectionary product produced mainly from wheat flour, water, yeast and salt by a series of process involving mixing, kneading, proofing, shaping and baking (Dewettinck et al., 2008). Bread has been a major component of human diet dating back to pre-historic man. This has made the baking of yeast leavened and sour dough bread one of the oldest biotechnological process (Christine et al., 2012). Bread is an important staple food, the consumption of which is steady and increasing in Nigeria. It is however relatively expensive, being made from imported wheat that is not cultivated in the tropics for climate reasons (Edema et al., 2005).

Cake is a baked batter made from sugar, egg, shortenings, milk and leavening mixed together in such a way as to produce a fluffy fined grained baked product (Victor et al., 1995). It is described as a desirable delicate tender, highly sweetened, non-yeast baked product (Okaka, 2005). Cake has become a constant food in our diet for a long time and their continual popularity has encouraged the development of newer and more attracttive product that is available in the market today (Eke et al., 2009). They can be served alone, packed with lunch, taken on a picnic or traditional favourite as refreshment for guests (Signori, 2004).

Chinchin is a fried snack popular in West Africa. It is a sweet, hard, donut-like baked or fried dough of wheat flour, and other customary baking items. *Chinchin* may also contain cowpeas (Akubor, 2004). Many people also bake it with ground nutmeg for flavor. It is usually kneaded and cut into small squares of 1 square inch or so, about a quarter of an inch thick, before frying (Mepha, 2007).

Wheat as the chief raw material in the production of wheat flour cannot thrive well in Nigerian soils; therefore wheat flour has to be imported. This leads to relatively high price of bakery goods. The availability of adequate supply of flour has been a major economic and political issue in Nigeria.

The objective of this work is to utilize sweet potato flour and starch as substitutes to wheat in bread making and confectionery as this may help to improve nutrition of consumers of root crops in Nigeria and also ensure national food security.

MATERIALS AND METHODS

Source of materials

Fresh roots of sweet potato variety TIS 87/0087 were harvested at about 8 weeks old after planting from the experimental trial of sweet potato Programme, National Root Crops Research Institute (NRCRI), Umudike, Nigeria.

Processing of sweet potato roots to flour

The method described by Etudaiye et al. (2008) with some modify-cations was employed. This involved washing of fresh roots, peeling, soaking (24 h to leach out sugars), decanting of water, sun-drying of chips, milling, sieving (with muslin cloth) to obtain fine flour and packaging.

Starch isolation from sweet potato roots

The method of Sanchez et al. (2005) with some modifications was used. This involved washing of fresh roots, peeling, grating, continuous washing with water (about 6 times),sedimentation of starch (6 h), decanting, sun-drying, milling and sieving (with muslin cloth) to obtained fine starch flour and packaging.

Development of wheat: sweet potato composites

The method of Etudaiye et al. (2008) was employed. Wheat flour was blended with sweet potato flour/starch as composites in the ratios: 80:20, 70:30, 60:40 and 50:50. Whole wheat flour, whole sweet potato flour and whole sweet potato starch were used as controls.

Preparation of recipes

The method of Aniedu and Oti (2008) was employed (Table 1).

Production of bread

The method recommended by Edema et al. (2005) with some modification was used. Whole wheat, sweet potato flour/ starch, and 80:20, 70:30, 60:40 and 50:50 blends of wheat: sweet potato flour/ starch were used. Samples were weighed based on 100 g standard. All ingredients as written in Table 1 were taken in a bowl and mixed at high speed with the aid of Philips hand mixer Type HR1453 for 10 min. The mixture was allowed to stand for 1 to 4 h at

Table 1. Preparation of recipes based on 100% wheat: sweet potato composite flours.

Ingredient	Bread	Cake	Chinchin
Sugar	1 table spoon	2 table spoon	1 table spoon
Margarine	1 table spoon	3 table spoon	½ table spoon
Yeast	½ table spoon	-	-
Salt	A pinch	-	-
Water	As required	-	-
Egg slurry	-	4 table spoon	2 table spoon
Baking powder	-	½ table spoon	1 table spoon
Grated nut meg	-	½ table spoon	½ table spoon
Vanilla essence	-	1 ml	-

Aniedu and Oti (2008).

room temperature for batter development. This was followed by gentle mixing for 5 min after which the batter was scaled into greased baking pans. Loaves were baked at 160 to 180°C for 35 min in an LG Gas cooker OMEGA 4B oven. After baking, the loaves were left for about 10 min in the oven. They were then quickly removed from the pans, arranged in trays and returned to the oven for 1 to 2 h or until required for analysis. Analyses were carried out after the baked loaves had attained room temperature or internal crumb temperature of about 35±2°C.

Production of cake

The method recommended by Okorie and Oyeneke (2012) with some modifications was used. Whole wheat, sweet potato flour/ starch, and 80:20, 70:30, 60:40 and 50:50 blends of wheat: sweet potato flour/ starch were used. The baking fat and granulated sugar were creamed together with the Kenwood mixer for 20 min until light. The eggs were beaten for 5 min with the homogenizer. This was done to prevent the curdling of batter. After batter development of a soft velvety feel, the vanilla essence (flavoring) was added. The mixed batter, each was mixed with milk and water to proportion and poured into greased cake pans. These were put in the oven and baked at temperature of 190°C for 15 min. The cakes were cooled and removed from the pan after 1 h. The cooled cake were packaged in aluminum foils and kept in shelf until required for sensory evaluation.

Production of *chinchin*

The method of Aniedu and Omodamiro (2012) with some modifications was used. Whole wheat, sweet potato flour/ starch, and 80:20, 70:30, 60:40 and 50:50 blends of wheat: sweet potato flour/ starch were used for the preparation of *chinchin*. Samples were weighed based on 100 g standard. Ingredients with the right measurements as reported on Table 1 were added with required water to form pastry samples. The pastry samples were spread on a board and cut into bits and the bits were cooked in deep hot oil until attractively brown in colour.

Functional properties and gelatinization temperature

Functional properties (water absorption capacity, oil absorption capacity, bulk density) and gelatinization temperature were determined by the methods of Etudaiye et al. (2008).

Determination of oil absorption (OAC) and water absorption capacities (WAC)

About 1 g of dry starch sample was weighed into 15 ml centrifuge tube and 10 ml of distilled water or oil was added. The sample was mixed thoroughly and allowed to stand for 30 min at room temperature and centrifuged at 2000-5000 rpm for 30 min. The volume of free water or oil (the supernatant) was read directly from the calibrated centrifuge tube. The amount of water or oil retained by the starch sample was from the difference in volume of the initial amount of water or oil added to that decanted after centrifugation.

Determination packed bulk density (BD)

About 50 g of dry starch sample was weighed into a granulated measuring cylinder and its volume was recorded. Next, the measuring cylinder was tapped constantly on the Table (10-15 times) until there was no further change in volume. Bulk density was calculated as the weight of the flour sample in grams per volume (g/ml).

Determination of gelatinization Temperature

About 10% of the starch sample was prepared in a test tube and the mixture was boiled with continuous stirring. The temperature was recorded 30 s after gelatinization was visually observed. Gelatinization temperature was taken with a thermometer and recorded in degree centigrade.

Sensory evaluation

Sensory evaluation was carried out with the method of Iwe (2002). Seven (7) points Hedonic scale was used where 7 = like extremely, 4 = neither like nor dislike and 1 = dislike extremely. Fourty (40) panelists comprising males and females who are regular consumers of confectioneries participated in the experiment. Bread, cakes and *chinchin* produced from 100% wheat flour were used as control.

Statistical analysis

Statistical analytical system (SAS) software, version 8, (2009) was used for data analysis. Analysis of variances was carried out and means separation was done using Fischer LSD to determine significant differences (P at 0.05%).

Tables 2. Functional properties of wheat : sweet potato and wheat: starch composite flour.

Sample	Wheat : sweet potato				Wheat : starch			
	Water absorption capacity (g/ml)	Oil absorption capacity(g/ml)	Gelatinization temperature (ºC)	Bulk density(g/ml)	Water absorption capacity (g/ml)	Oil absorption capacity(g/ml)	Gelatinization temperature (ºC)	Bulk Density (g/ml)
100% wheat	2.0^{b}	2.0^{a}	58.00^{d}	0.78^{b}	2.0^{a}	2.0^{a}	58.0^{b}	0.78^{a}
80:20	2.0^{b}	2.0^{a}	60.25^{d}	0.82^{a}	2.0^{a}	1.5^{b}	54.3^{c}	0.78^{a}
70:30	2.0^{b}	2.0^{a}	70.25^{a}	0.73^{c}	1.5^{b}	2.0^{a}	58.0^{b}	0.74^{b}
60:40	2.0^{b}	1.5^{b}	60.25^{c}	0.68^{d}	1.5^{b}	1.5^{b}	65.3^{a}	0.74^{b}
50:50	2.5^{a}	2.0^{a}	60.45^{c}	0.68^{d}	1.5^{b}	1.5^{b}	65.5^{a}	0.77^{a}
100% Sweet potato	2.5^{a}	2.0^{a}	49.00^{e}	0.69^{d}	1.5^{b}	1.0^{c}	48.0^{d}	0.77^{a}
LSD (0.5%)	0.02	0.03	1.54	0.01	0.02	0.02	0.87	0.01

Mean values down the columns with the same alphabet are not significantly different (P>0.05). 80:20, 70:30, 60:40 and 50:50= wheat: sweet potato blends.

RESULTS AND DISCUSSION

Table 2 shows the functional properties of sweet potato flour and starch and their composites with wheat flour. Values of water absorption capacity, oil absorption capacity, gelatinization temperature and bulk density were within the range of previous values reported by Etudaiye et al. (2008) in a work titled functional properties of wheat: sweet potato composite flour and sensory qualities of confectioneries produced from the composites. Also, values of water absorption capacity were in line with that of Ojinaka et al. (2009) in cocoyam starch modification effects on functional, sensory and cookies qualities which ranged from 0.83-1.67 g/ml. Gelatinization temperature (GT) of the flours and starches and their blends with wheat flour were within the ranges of values reported by Woolfe (1992) where GT ranged from 58-69°C, 58-75°C and 65-80°C. GT is a measure of consistency of a starch suspension when it is heated at a certain temperature for a given period of time. It enhances the body and texture of a product (Onimawo et al., 1998). High gelatinization gives a good and easier cooking quality than low gelatinization. However, high gelatinization temperature may require more heat energy and costs. This suggests that flours and starches of the sweet potato variety should not be added to a formula where gelling is required below 58ºC. Bulk density (BD) of wheat: sweet potato starch composites gave higher values than the previous report made by Etudaiye et al. (2008) but was within the range of values 0.62-0.75 g/ml of Ojinaka et al. (2009). BD is an indication of porosity of a product which influences package design. It is affected by moisture content and particle size distribution of the flour (Onimawo et al., 1998).

High bulk density of the of flours and starches indicate that they would serve as good thickeners in food products (Adebowale et al., 2005), while the low bulk density of flours and starch samples will be suitable for the formulation of high nutrient density weaning food (Mepba et al., 2007).

Tables 3 shows that up to 40% inclusion of sweet potato starch gave acceptable bread. This is an advantage over the recommended 10% inclusion of cassava flour with wheat in bread making in Nigeria. The process modification improved the colours of the cake sample (Though, not a primary focus in the study) (Table 4). Acceptable cakes up to 60:40 wheat:sweet potato starch composite and 50:50 wheat:sweet potato composites flour were achieved as compared with cakes prepared from 80:20 wheat:sweet potato composite flour in the work reported by Etudaiye et al. (2008).

Table 5 shows that general acceptability of *chinchin* prepared from 80:20 and 50:50 wheat: sweet potato starch composites were acceptable by the panelists. However, *chinchin* produced from wheat: sweet potato composite flour (Table 5) showed general acceptability up to 50:50 ratios. Colours of products from composites up to 50:50 ratios were also acceptable and showed no significant difference (P>0.05).

Conclusion

The study shows that sweet potato flour and starch gave acceptable products: cake, *chinchin* and bread. They also showed appealing, desirable

Tables 3. Sensory evaluation of Bread produced from wheat: sweet potato starch and flour composite.

Sample	Starch					Flour				
	Colour	Taste	Texture	Aroma	General acceptability	Colour	Taste	Texture	Aroma	General acceptability
100% Wheat	6.0^{a}	6.4^{a}	6.2^{a}	6.1^{a}	6.1^{a}	5.9^{a}	5.6^{a}	6.1^{a}	5.9^{a}	6.0^{a}
80:20	5.9^{a}	$5.4a^{b}$	5.6^{ab}	5.3^{ab}	5.3^{ab}	5.5^{a}	4.8^{ab}	5.4^{a}	5.1^{ab}	5.0^{ab}
70:30	5.4^{ab}	4.9^{bc}	5.4^{ab}	5.1^{ab}	5.1^{ab}	4.9^{ab}	4.0^{bc}	4.3^{b}	4.3^{bc}	4.0^{bc}
60:40	5.0^{b}	4.7^{bc}	5.6^{ab}	5.2^{ab}	5.0^{b}	4.1^{bc}	3.3^{cd}	3.8^{d}	3.6^{cd}	3.3^{cd}
50:50	4.0^{c}	4.0^{e}	4.6^{b}	4.4^{bc}	4.3^{b}	3.4^{cd}	2.5^{d}	3.4^{b}	2.9^{de}	3.1^{cd}
100% Sweet potato	2.8^{d}	2.4^{d}	2.9^{c}	3.3^{c}	2.9^{c}	2.6^{d}	2.4^{d}	2.1^{c}	2.3^{e}	2.3^{d}
LSD (0.5%)	0.99	1.1	1.2	1.1	1.1	1.2	1.0	1.1	1.1	1.0

Mean values down the columns with the same alphabet are not significantly different (P>0.05). 80:20, 70:30, 60:40 and 50:50= wheat: sweet potato blend.

Tables 4. Sensory evaluation of Cakes produced from wheat: sweet potato starch and flour composite

Sample	Starch					Flour				
	Colour	Taste	Texture	Aroma	General acceptability	Colour	Taste	Texture	Aroma	General acceptability
100% wheat	6.0^{a}	5.9^{a}	5.5^{ab}	5.7^{a}	5.8^{a}	6.3^{a}	5.7^{a}	5.7^{a}	5.8^{a}	6.1^{a}
80:20	5.4^{a}	5.6^{ab}	5.6^{a}	5.4^{ab}	5.8^{a}	5.5^{b}	5.7^{a}	5.0^{b}	5.5^{ab}	5.4^{b}
70:30	5.3^{a}	5.6^{ab}	5.7^{a}	5.7^{a}	5.5^{ab}	5.8^{ab}	5.6^{ab}	5.2^{ab}	5.5^{ab}	5.8^{ab}
60:40	5.3^{a}	5.5^{ab}	5.7^{a}	5.3^{abc}	5.8^{a}	5.1^{bc}	5.2^{ab}	4.9^{bc}	5.1^{abc}	5.1^{bc}
50:50	4.3^{b}	5.1^{b}	5.0^{ab}	4.8^{c}	5.2^{ab}	5.6^{ab}	5.3^{ab}	4.9^{bc}	4.9^{bc}	5.4^{b}
100% Sweet potato	3.9^{b}	5.0^{b}	4.8^{b}	5.0^{bc}	4.9^{b}	4.4^{c}	4.9^{b}	4.2^{c}	4.7^{c}	4.7^{c}
LSD (0.5%)	0.8	0.7	0.7	0.6	0.6	0.8	0.7	0.7	0.7	0.7

Mean values down the columns with the same alphabet are not significantly different (P>0.05). 80:20, 70:30, 60:40 and 50:50= wheat: sweet potato blends.

Tables 5. Sensory evaluation of *Chin-Chin* produced from wheat: sweet potato starch and flour composite.

Sample	Starch					Flour					
	Colour	Taste	Texture	Aroma	General acceptability	Colour	Taste	Texture	Crispiness	Aroma	General acceptability
100% wheat	5.96^{a}	5.76^{a}	5.7^{a}	5.8^{a}	5.5^{a}	5.5^{a}	5.6^{a}	5.5^{a}	5.4^{a}	5.3^{a}	5.9^{a}
80:20	5.7^{a}	5.2^{ab}	5.2^{ab}	5.1^{ab}	5.1^{abc}	5.1^{a}	5.1^{a}	5.2^{a}	5.2^{a}	4.8^{ab}	4.9^{ab}
70:30	5.2^{ab}	4.4^{bc}	4.4^{bc}	5.2^{a}	4.4^{bcd}	5.2^{a}	4.9^{a}	5.1^{a}	4.9^{a}	4.7^{ab}	5.1^{a}

Table 5. Contd

60:40	4.3^{bc}	4.3^{bc}	4.2^{c}	4.2^{bc}	4.2^{cd}	5.6^{a}	5.6^{a}	5.2^{a}	5.2^{a}	5.3^{a}	5.3^{a}
50:50	5.5^{a}	5.2^{ab}	5.4^{a}	5.1^{ab}	5.2^{ab}	5.0^{a}	4.9^{ab}	4.9^{ab}	4.7^{ab}	4.9^{ab}	5.1^{a}
100% Sweet potato	3.7^{c}	3.8^{c}	3.6^{c}	4.0^{c}	3.5^{d}	3.6^{b}	4.0^{b}	4.0^{b}	3.7^{b}	4.0^{b}	4.0^{b}
LSD (0.5%)	1.0	0.92	0.9	1.98	1.0	1.0	0.9	1.0	1.0	1.0	1.0

Mean values down the columns with the same alphabet are not significantly different (P>0.05). 80:20, 70:30, 60:40 and 50:50= wheat: sweet potato blends.

and acceptable colours except in products from 100% sweet potato flour and starch. Flours and starches blended with wheat flour at different ratios gave good functional properties which enhanced their utilization potentials. This work recommends the promotion and utilization of sweet potato flour and starch in bread making, preparation of cake and *chinchin* in Nigeria, that is known to fully depend on imported wheat for bread and other confectionery production. Furthermore, the utilization of sweet potato will help to improve nutrition of consumers of root crops in Nigeria and also ensure national food security.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Screening model for indolocarbazole-producing microorganisms by molecular probe combined with high performance liquid chromatography (HPLC) method

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Indolocarbazole (ICZ) alkaloid constitutes a group of natural products which manifested powerful biological activity, especially anticancer. However, it was laborious to find ICZs-yield organism when anticancer activity was adopted as selection target. The specificity of 6 primer pairs was detected and the results revealed that primer rebBN1/rebBC1 possessed preferable specificity because indolocarbazole-producers DNA was exclusively amplified and no target fragment was amplified from any of indolocarbazole nonproducers. Therefore, primer rebBN1/rebBC1 could specifically identify indolocarbazole-producers from microorganism strains.

Key words: Indolocarbazole, rebBN1/rebBC1, ICZs-producing microorganism screening.

INTRODUCTION

The indolocarbazole (ICZ) family is an important class of natural products isolated from bacteria, actinomycetes, cyanobacteria, fungi, slime moulds and marine invertebrates (Sanchez et al., 2006). Since the discovery of staurosporine as the first ICZs in 1977, a variety of ICZs (more than 120) have been found and attracted great attention of chemists, biologists, physicians and pharmaceutical for their biological activities and the prospective number of derivatives from the basic backbone alone. Indolocarbazole (ICZs) compounds are structurally characterized by possessing an indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole core derived from two units of tryptophan, with sugars attached derived from glucose and methionine. According to their structural features and mechanisms of actions, ICZs are divided into two major classes (Gribble and Berthel, 2001). The first class was characterized by a sugar moiety linked to both indole nitrogen atoms of the indolocarbozole core to form a

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Abbreviations: ICZ, Indolocarbazole; **REB,** rebeccamycin; **STA,** staurosporine; **CTAB,** cetyl trimethyl ammonium bromide; **PCR,** polymerase chain reaction; **DMSO,** dimethyl sulfoxide ; **BSA,** bovine serum albumin; **EtOAc,** ethyl acetate; **HPLC,** high performance liquid chromatography; **ORFs,** open reading frames.

"closed" indol-carbazole and showed inhibition to protein kinase C, such as K-252a and staurosporine (STA). The second class contains a sugar moiety attached only to one of the indole nitrogen atoms of the aglycone by a β-glycosidic linage to form an "open" bisindolymaleimide and exhibit inhibition to DNA topoisomerase I, such as rebeccamycin (REB) and AT2433. Despite the differences, it is assumed that the presence of the sugars in both classes appears to be essential for the biological activity of ICZ compounds (Bailly et al., 1999).

ICZs display a wide range of biological activities including antibacterial, antifungal, antiviral, hypotensive, and neuro-protective activities. However, the most attracting point is their therapeutic potential as anti-cancer drugs. Recently, their strong effects to various harmful insects were discovered by our team (related article under review). Although several ICZs derivatives have already entered clinical trials for the treatment of cancer or other diseases, no analog has yet been launched onto the market and great efforts have been made to find or generate ICZs derivatives with improved properties for drugs. Despite the abundant diversity of ICZs-origin organisms, microbes were the main origin of most known ICZs. During the search for novel ICZs-producing organisms and the isolation of ICZs, blindness and repetitiveness have been the formidable obstacles with great loss of labor and time. In order to solve the problem, the feasibility of using molecular probes combined with HPLC to detect ICZs-producing microorganisms was investigated in this article.

MATERIALS AND METHODS

Strains in this research

Totally, 56 strains with potential pharmaceutical activity, including bacteria 10, actinomycetes 36, and fungi 10 deposited in China Pharmaceutical Marine Microbiological Databank (which was established by our team) were selected as test microbes. Among them, *Streptomyces nitrosporus* CQT14-24 (T14-24) was the staurosporine-producing strain which was under intensive investigation in our lab. This strain was isolated from sediment of Chukchi Sea (the North Pole) and were deposited both in China Pharmaceutical Marine Microbiological Databank (No.HTTMS-F04008) and China General Microbiological Culture Collection Center (No.CGMCC 4607), and recorded at GenBank with the accession number FJ821473. *S. nitrosporus* CQT5-L25-1 (5-L25-1) was a mutant of T14-24 with increased yield of staurosporine and increased kinds of ICZs homolog. T14-24 and 5-L25-1 were used as positive control.

Culture conditions and DNA isolation

For ICZs extraction, bacteria, actinomycete and fungi strains were shaking cultured by using MYPD medium, Gauze No.1 medium, and MPDA medium at 25°C for 7 days, 12 days and 10 days respectively. For DNA extraction, strains were cultured by using the above mentioned medium for 2 days. Bacteria and actinomycete DNA were isolated by standard cetyl trimethyl ammonium bromide (CTAB) method while fungi DNA was isolated through glass beads method (Alessandro et al., 2001).

Molecular probes adopted in this article

Different conservative sequences of ICZs gene cluster were sought among known literatures and selected primers were listed as Table 1. Primers rebBN1 / rebBC1 were initially designed to amplify *rebD* of rebeccamycin gene cluster from *Streptomyces* sp. TP-A0274 (Onaka et al., 2002). Primers CS035 / CS036 were designed to carry out *in situ* colony hybridization of a constructed cosmid library of *Streptomyces longisporoflavus* DSM10189 which was highly similar to *rebD* in *Lechevalieria aerocolonigenes* (Salas et al, 2005). Primers StaCN / StaCH and StaPN /StaPH were designed to amplify *staC* and *staP* in *L. aerocolonigenes* (Howard-Jones et al., 2006). Primers RebD1 / RebD2 were designed to amplify *rebO* in *L. aerocolonigenes* ATCC 39243 (Howard-Jones et al., 2005). Primers NGT1 / NGT2 were designed to amplify *ngt* (now named *rebG*) in *L. aerocolonigenes* ATCC 39243 (Onaka et al., 2003).

Polymerase chain reaction (PCR) amplification

PCR amplifications were performed in 50 μl containing 1 μl template, 1 μl of each primer (10 μmol/L), 1 μl deoxynucleotide triphosphates (2.5 mmol/L), 5 μl 10× polymerase chain reaction (PCR) buffer, 1.5 μL $MgCl_2$ (1.5 mmol/L), 1.5 μL dimethyl sulfoxide (DMSO), 1.5μL bovine serum albumin (BSA) (0.1%), 0.25μL TaqDNA polymerase (2U). The cycling parameters were: initial denaturation for 5 min at 95°C followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, and a terminal extension step of 72°C for 15 min.

Isolation of ICZs

After fermentation, the culture broth of different strains was filtered through cheesecloth to separate it into supernatant and mycelia; the former was extracted with equal volume ethyl acetate (EtOAc) for two times while the later was extracted with EtoAc (200ml) by supersonic method; both of the EtOAc solutions were concentrated under reduced pressure to afford an MeOH solutions (5 ml). The concentration of indolecarbazole alkaloids in MeOH solutions was determined by high performance liquid chromatography (HPLC) method. The analytical HPLC system was composed of Waters Acquity UPLC and BEH c18 column (1.7 μm, 2.1 × 5 0mm); chromatographic condition: (0 to 9 min, 5%-100% MeOH, 9-11 min, 100% min, 0.5 ml/min).

RESULTS

ICZs producing microorganisms

Among 54 test strains, four strains (all actinomycetes) including *Streptomyces* sp. T9-33, *S. nitrosporeus* T13-12, *Streptomyces* sp. YH3-2, and *Streptomyces* sp. YT1-28 were detected to yield ICZs compounds while other strain showed no production of ICZs. Therefore, there were totally 6 strains (T14-24 and 5-L25-1 as positive control) which produced ICZ compounds. The HPLC spectrum of the six strains was similar with difference of

Table 1. Primers used in this study.

Primer name	Primer sequence	Reference
rebBN1	5'-GAAGAATTCGTSATGCTSCAGTACCTSTA-3'	Onaka et al. (2002)
rebBC1	5'-CGAAAGCTTSAGGAASAGGTGGTGCTCSCC-3'	
CS035-StaD	5'-ATATAAGCTTGATGGCCCAGCACTTCGG-3'	Salas et al. (2005)
CS036-StaD	5'-TATCTAGACGGCGGGCGGAAGCGGTC-3'	
RebD1	5'-GGAGAGCATATGAGCGTCTTCGACCTG-3'	Howard-Jones et al. (2005)
RebD2	5'-GTCAAGCTTTCGCGGTCCTTCCGTTGC-3'	
StaPN	5'-GGAGAGCATATGCCATCCGCGACGCTGC-3'	Howard-Jones et al. (2006)
StaPH	5'-GTCAAGCTTGGGGTGGCTGGCCGAGGG-3'	
StaCN	5'-GGAGAGCATATGACGCATTCCGGTGAGCGGACC-3'	Howard-Jones et al. (2006)
StaCH	5'-GTCAAGCTTGCCCCGCGGCTCACGGGGCGCGGC-3'	
NGT1	5'-TCGGAATTCATGGGGGCACGAGTGCTG-3'	Onaka et al. (2003)
NGT2	5'-AGGAAGCTTGAACGGGCCGACGAACCT-3'	

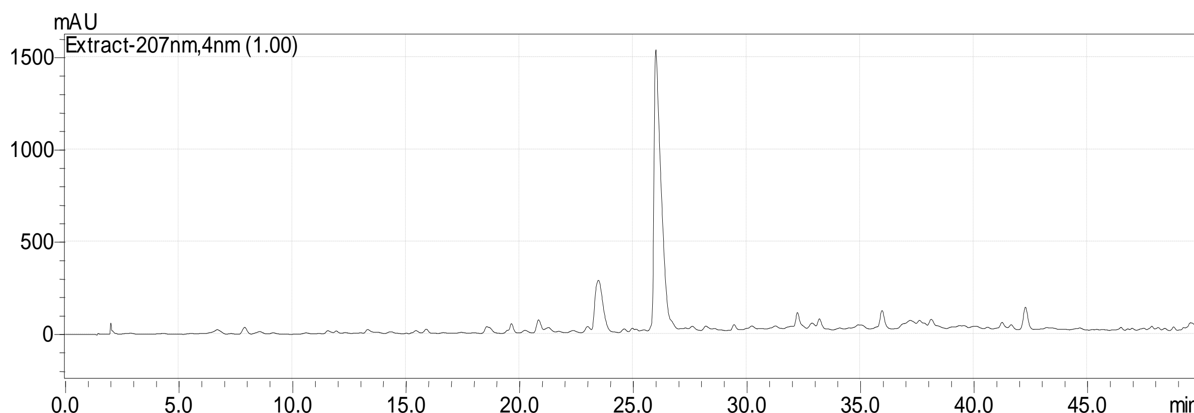

Figure 1. High performance liquid chromatography (HPLC) chromatograph of ICZs producing microorganisms 5-L25-1.

peak area. Among them, strain 5-L25-1 yielded the maximum amount of ICZ (peak area 17089081) while strain YH 3-2 yielded the minimum of ICZ (peak area 1311480) (Figure 1). The ultraviolet absorption spectrum of the 5 strain except 5-L25-1 was similar with the main component of staurosporine. As for 5-L25-1, the ultraviolet absorption spectrum showed two main components staurosporine and K252c (Figure 2).

The specificity of different primers

Different open reading frames (ORFs) fragments were amplified using specific primers and the results showed that for almost primers except StaPH/StaPN visible fragments were amplified only when chromosomal DNAs from indolocarbazole producers were used as templates. However, there was no regularity and specific indicator band(s) among the fragments bands except primers rebBN1/rebBC1 (Figure 3, A-E lane 1-6).

The specificity of primers rebBN1/rebBC1

The primers, rebBN1/rebBC1, were synthesized using the highly conserved regions corresponding to amino acids from 611 to 618 and from 780 to 786 in RebD amino acid sequence and were supposed to produce fragment of 0.5 to 0.7 kb. As shown in Figure 2F, a 0.5 kb and/or 0.75 kb fragments were amplified only when chromosomal DNAs from indolocarbazole producers were used as templates (Figure 3F; lane 1-6). On the other hand, any DNA fragment with the size of neither 0.5 kb nor 0.75 kb was amplified with chromosomal DNAs from indolocarbazole nonproducers. The results demonstrate that this primers pairs possessed preferable specificity because indolocarbazole-producers DNA was exclusively amplified. Since no target fragment was amplified from any of indolocarbazole nonproducers, this primer pair could specifically identify indolocarbazole-producers from strains.

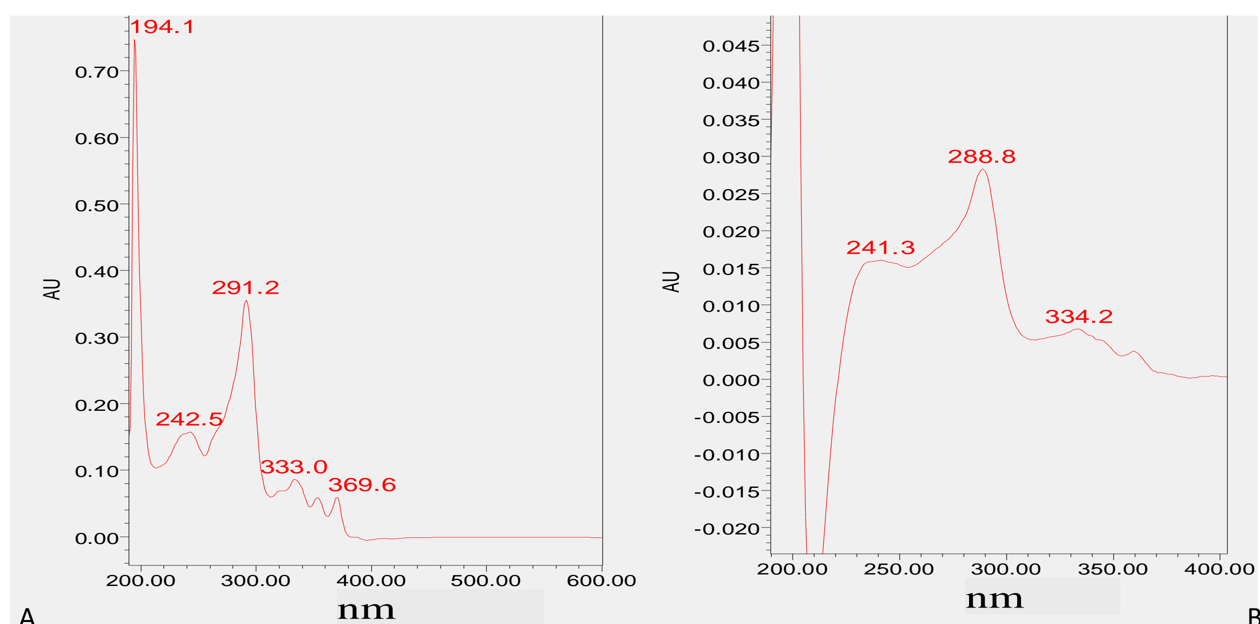

Figure 2. UV spectrum of ICZs producing microorganisms 5-L25-1.A, Staurosporine; B, K252c.

DISCUSSION

ICZs have attracted the attention of many researchers from different disciplines for their biological activities especially anticancer property. This property may be due to different mechanisms of action, including inhibition of protein kinases, DNA intercalation, and inhibition of DNA topoisomerases. Protein kinases and DNA topoisomerases are important in cell proliferation so specific inhibitors are promising candidates for antitumor or antifungi drugs and pesticide to kill harmful insects. Despite the high inhibition activities, certain known ICZs also exhibited high toxicity with low specificity which limited their direct clinical application. Therefore, great efforts have been made to search or generate novel ICZs derivatives with higher inhibitory specificity. Although organic chemistry methods are routinely used to synthesize and modify natural products, harvesting the product (or a modifiable precursor) from the natural source is often the only cost-effective way of production. Despite the abundant diversity of ICZs-origin organisms, most ICZs are isolated from actinomycetes: rebeccamycin is from *L. aerocolonigenes* ATCC 39243 (Nettleton et al., 1985), staurosporine is from *L. aerocolonigenes* subsp. *staurosporeus* and *Streptomyces* sp. strain TP-A0274 (Omura et al., 1977), K252a is from *Nocardiopsis* sp. strain K252 (Kase et al., 1986). Herein, actinomycetes were considered as the main test strains in this research along with certain bacteria and fungi strains and our results also verified this phenomenon. Among 54 test strains, 4 ICZs-yielding strains were all actinomycetes while no bacteria and fungus strains was detected to produce ICZs. Although the searching scope has been narrowed mainly to actinomycetes during the search for natural novel ICZs, it was still laborious to find ICZs-yield organism when anticancer activity was adopted as selection target. Therefore, in order to reduce the blindness, specific molecular probes might be feasible in effectively narrowing screening strains scope considering the fact that ICZs were biosynthesized through similar pathways and encoded by corresponding biosynthesis gene cluster.

Up to now, although the basic biosynthetic pathway for the indolocarbazole aglycone was revealed in the analysis of rebeccamycin and staurosporine biosynthesis, modified pathways for the individual indolocarbazole compounds remain unidentified. Studies on the biosynthesis of ICZs have shown that the indolocarbazole core is formed by decarboxylative fusion of two tryptophan-derived units, whereas the sugar moiety is derived from glucose (Pearce et al., 1988; Meksuriyen and Cordell, 1988). Biosynthetic studies through isotope-labeled precursors indicate that the rebeccamycin and staurosporine indolocarbazole cores are derived from L-tryptophan (L-Trp) via a series of oxidative transformations. The initial pathways to the two aglycones follow very similar routes, differing only by the oxidation state at the C-7 position of aglycone and the pattern of connectivity between the deoxysugar and aglycone. Subsequent *N*-glycosylation and tailoring modifications follow divergent pathways toward rebeccamycin and staurosporine (Howard-Jones et al., 2006). Biosynthesis of ICZs can be divided into five stages catalyzed by different sets of enzymes, which are(*i*) tryptophan modification (halogenation) by RebH;

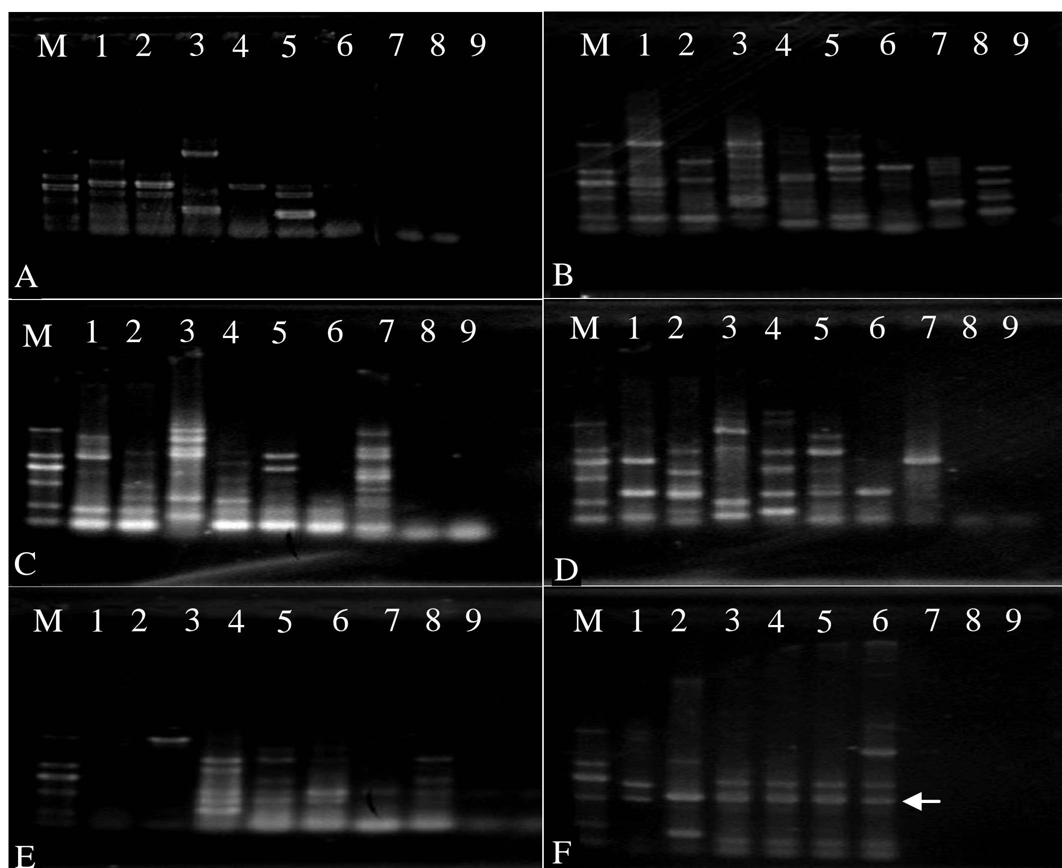

Figure 3. Sensitivity of different primers for detection of ICZs producing microorganisms. A, Primers of RebD1/RebD2; B, primers of StaPH/StaPN; C, primers of StaCN/StaCH; D, primers of CS035/CS036; E, primers of NGT1/NGT2; F, primers of rebBN1/rebBC1. Lane M, DNA Marker 3 ; lane 1-9, different strains of T14-24, 5-L25-1, 3-2, T13-12, T1-28, T9-33, Fc02, T9-37, G19-24, respectively.

(*ii*) dimerization by RebO/RebD or StaO/StaD; (*iii*) decarboxylative ring closure by RebC/RebP or StaC/StaP; (*iv*) glycosylation by RebG or StaG; and (*v*) sugar modification by RebM or several enzymes for staurosporine before and/or after glycosylation (refs. 10–13 and this article). Stages *ii* and *iii* constitute the central reactions in the pathway, whereas the other stages can be considered as accessories for "tailoring" the alkaloid skeleton (Sanchez et al., 2005). In this sense, RebO/RebD (or StaO/StaD) and RebC/RebP (or StaC/StaP) play crucial role in the formation of the ICZs molecule backbone. On the other hand, some researchers consider glycosyl in ICZs is essential for its biological activities (Sanchez et al., 2005). Therefore, we focus our intention also on genes encoded these two enzymes.

In recent years, an increasing amount of information has become available on the molecular genetics of indolocarbazole biosynthesis. Until now, four kinds of ICZs biosynthetic gene clusters including rebeccamycin, staurosporine, AT2433, and K252a were cloned. The genetic organization of these four indolocarbazole gene clusters is quite similar and all of them contain genes required for the biosynthesis of the aglycone, biosynthesis and transfer of the deoxysugar and regulation. The rebeccamycin biosynthesis gene cluster was taken as an example. On the basis of sequence analysis and database searches about rebeccamycin gene cluster from *L. aerovolonigenes* ATCC 39243, Sanchez et al. (2002) proposed that there are 11 ORFs including four for ICZ-core biosynthesis (*rebO*, *rebD*, *rebC* and *rebP*), two for halogenation (*rebH* and *rebF*), glycosylation (*rebG*, the name was changed from *ngt*), and sugar methylation (*rebM*), as well as a regulatory gene (*rebR*) and two resistance and secretion genes (*rebU* and *rebT*). There were approximately counterparts in other three gene clusters. Considering the conservation in related gene clusters, gene fragment of different ORFs were adopted as molecular probe to detect ICZs producing microorganisms among bacteria and fungi strains which was deposited in our lab. To be specific, primers rebBN1/rebBC1 and CS035/CS036 were designated to amplify *rebD/StaD* gene with about

0.3 kb/0.5 kb fragment. In the biosynthesis of indolocarbazoles, chromopyrrolic acid or 11,11'-dichlorochromopyrrolic acid is presumed to be an intermediate which might be directly biosynthesized by *rebD* which encodes a putative chromopyrrolic acid synthase (Onaka et al., 2002; Salas et al., 2005). Primers RebD1/RebD2 were designed to amplify *rebO* fragment which was predicted to be a flavoprotein encode gene (Howard-Jones et al., 2005). Primers StaPN/StaPH and StaCN/StaCH were designed to amplify *staP* and *staC*, respectively (Howard-Jones et al., 2006). RebP and RebC are responsible for the oxidative decarboxylation and ring fusion reactions that create the six-ring indolopyrrolocarbazole rebeccamycin aglycone (Sanchez et al., 2005). The RebG (or StaG, once named Ngt) protein converted an indolocarbazole to its N-glucoside indicating that *RebG* (or *StaG*, once named *ngt*) is responsible for N-glycosylation in rebeccamycin biosynthesis (Onaka et al., 2003). An internal fragment of *ngt* was usually adopted to clone rebeccamycin biosynthetic genes as a probe. Based on these information, 6 primer pairs were selected to amplify different ORF fragment for detection of ICZs-yield microorganisms. The results reveal that there were varied conservation degree among different ORFs, to be specific, *staD* (or *rebD*) possesses the maximum of conservation which may be owe to chromopyrrolic acid synthase which is the key enzyme in the indolocarbazole aglycone synthesis and thus its existence is expected to be a useful indicator of indolocarbazole-producing strains. However, the conservation degree of different fragments in this ORF varied according to our results. Primer pair CS035/CS036 and rebBN1/rebBC1 were both designed to amplify DNA fragment in *StaD* (or *rebD*), with designated region of 1 to 323 and 611 to 1110, respectively. The results reveal that fragment amplified by primer pair rebBN1/rebBC1 showed preferable conservation among ICZs-yielding microorganisms. In addition, since chromopyrrolic acid (or 11,11'-dichlorochromopyrrolic acid) might be the common intermediate of different kinds of ICZs, the primer pair could theoretically detect microorganism producing each kind ICZs.

On the other hand, although glycosylation is very important because biological activity of ICZs requires the presence of the sugar, the primer pair to amplify gene encode for glycosyltransferase *RebG* (or *StaG*, once named *ngt*) showed low specificity to detect ICZs-yielding microorganisms. This might be because of the major structural difference between the two kinds of ICZs: either through a single nitrogen (in the case of rebeccamycin) or through two nitrogen and this property might lead to the flexibility of glycosyltransferase which makes *RebG* (or *StaG*) improper probe region.

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African Journal of Biotechnology

Full Length Research Paper

Identification and characterization of putative conserved IAM-hydrolases in developing rice grains

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Indole-3-acetamide (IAM) pathway was proposed to produce indole-3-acetic acid (IAA). This pathway was proven to be functional in many species of bacteria and expected to operate in plants based on the identification of IAM and IAM-hydrolase (AMI) activity in certain plant species. Available putative AMI sequences from a wide array of monocot and dicot plants were identified and the phylogenetic tree was constructed and analyzed. We identified in this tree, a clade that contained sequences from species across the plant kingdom suggesting that AMI is conserved and may have a primary role in plant growth and development. This clade contained the isolated and well characterized *Arabidopsis* and *Nicotiana* AMIs. The preliminary reverse transcriptase polymerase chain reaction (RT-PCR) results for the conserved rice AMI (Os04G02780) showed a transcript for the gene encoded by this enzyme at 1, 7 and 21 days after anthesis (DAF) during the active period of IAA accumulation in developing grains. Based on the accumulated data, we suggested that sequences in this clade are conserved and have an important role in IAA synthesis or other primary processes in plant development.

Key words: Auxin, indole-3-acetic acid, indole-3-acetamide, tryptophan, IAM-hydrolase.

INTRODUCTION

Auxin, from the Greek word "auxein" which means to grow, is found in animals, plants, fungi, and bacteria but having growth regulation effects only in plants. Auxin is an essential plant hormone affecting virtually all aspects of plant growth and development. It affects cell division, elongation, embryogenesis, fruit development, organ patterning, root growth, lateral root initiation, leaf expansion, shoot architecture, and vascular development. Despite the century-long history of auxin research, the complete picture of how plants synthesize IAA is far from complete understanding. Two major pathways for IAA production have been proposed: the tryptophan (Trp)-dependent pathways and a Trp-independent one. Tryptamine pathway, indole-3-pyruvic acid pathway (IPA), indole-3-acetaldoxime (IAOx) pathway and IAM pathway were proposed to convert Trp to IAA. Plant genes implicated in IAA biosynthesis as well as the reactions catalyzed by the encoded enzymes are illustrated in Figure 1.

The IPA pathway has recently fully elucidated and proven functional in *Arabidopsis* (Mashiguchi et al., 2011; Won et al., 2011). In this pathway, a tryptophan aminotransferase (TAA) converts Trp to IPA whereas; YUCCA, A flavin monooxygenase, catalyzes the conversion of

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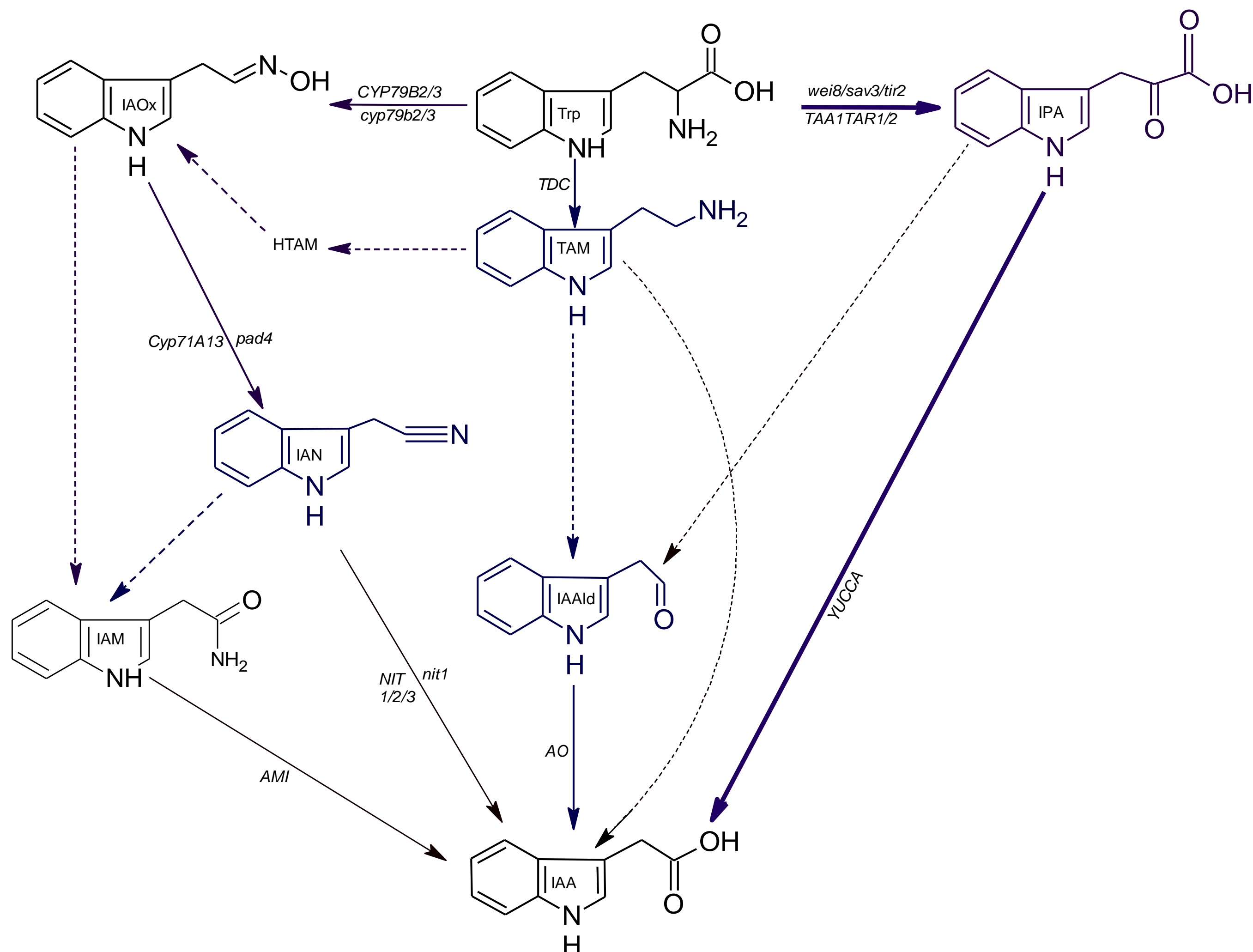

Figure 1. Proposed routes from tryptophan to IAA in plants. Bold lines refer to the first complete dissected pathway of IAA synthesis in plants. Dashed lines indicate that neither a gene nor enzyme activity has been identified in any member of plants. Trp: tryptophan; IAOx: Indole-3-acetaldoxime; IAM: indole-3-acetamide; IAA: indole-3-acetic acid; IPA: indole-3-pyruvic acid; TAM: tryptamine; HTAM: N-hydroxyl tryptamine; IAAld: indole-3-acetaldehyde; IAN: indole-3-acetonitrile; TAA: tryptophan aminotransferase; AMI: amidase; NIT: nitrilase; AO: aldehyde oxidase; YUCCA: a flavin monooxygenase. References for mutant phenotypes are described in Stepanova et al., 2008 (*wei8*), Tao et al., 2008 (*sav3*), Yamada et al., 2009 (*tir2*), Zhao et al., 2002 (*cytp79b2/3*); Nafisi et al., 2007 (*pad4*) and Normanly et al., 1997) (nit1). Gene abbreviations are given in upper case italics. Mutant alleles are given in lower case italics.

IPA to IAA. IAA produced via IPA pathway was found to have a critical role in various developmental processes including embryogenesis, flower development, seedling growth, vascular patterning, lateral root formation, tropism, shade avoidance, and temperature-dependent hypocotyl elongation (Dai and Zhao, 2006, 2007; Stepanova et al., 2008; Tao et al., 2008).

The mutants in TAA or YUCCA of *Arabidopsis* are not lethal suggesting that other pathways together with the IPA are expected. The mutant in Vanishing tassel2 (Vt2), which encode grass-specific TAA, was reported to account for 60% reduction in IAA level (Phillips et al., 2011). This hormone in plant is crucial and two pathways of IAA production or more may be needed to ensure the efflux of enough amount of IAA. Moreover, multiple IAA biosynthetic pathways may contribute to regulation of IAA production. Moreover, two pathways of IAA synthesis in bacteria have been fully elucidated.

Indole-3-acetamide has also been proposed to be an IAA precursor in higher plants. The occurrence of IAM as an endogenous compound was reported in young fruits of *Citrus unshiu* (Takahashi et al., 1975), hypocotyls of Japanese cherry (Saotome et al., 1993), seedlings of *A. thaliana*, coleoptiles of rice and maize as well as apexes of tobacco (Sugawara et al., 2009). IAM was also detected in a sterile-grown *Arabidopsis thaliana* and the

microbial contributions were found to be insignificant (Pollmann et al., 2002). Pollmann et al. (2009) suggested the role of IAM as an intermediate in IAA synthesis based on the observation that Trp is converted to IAA via IAM in the cell-free *Arabidopsis* extract.

The enzyme that catalyze the conversion of Trp to IAM is unknown, but the gene encodes IAM-hydrolase which converts IAM to IAA, has been initially cloned and characterized in *A. thaliana* (Pollmann et al., 2003), and *Nicotiana tabacum* (Nemoto et al., 2009). IAM-hydrolyzing activity was also observed in wild and cultivated rice as well as cell-free extracts from various tissues of trifoliata orange *(Poncirus trifoliata* Rafin) (Kawaguchi et al., 1993; Kawaguchi et al., 1991). AMI was partially purified from rice cells and found to hydrolyze IAM, IAA ethyl ester in addition to the IAM homologue, 1-naphthalene-acetamide (Arai et al., 2004).

The indole-3-acetamide pathway of auxin biosynthesis has been worked definitely in bacteria. Tryptophan-2-monooxygenase, encoded by the *iaam* gene, catalyses the first step in this reaction converting Trp to IAM. The second step (IAM to IAA) is catalyzed by IAM-hydrolase that encoded by the *iaaH* gene. This pathway was found to be operated in a number of bacterial species including *Agrobacterium* (Inze et al., 1984), *Azospirillum* (Bar and Okon, 1993), *Streptomyces* (Manulis et al., 1994), methylobacteria, *Bradyrhizobium* (Sekine et al., 1989), *Rhizobium* (Theunis et al., 2004), *Pantoea agglomerans* (Manulis et al., 1998), and *Pseudomonas* (Magie et al., 1963).

A high degree of similarity between IAA biosynthesis pathways in plants and bacteria was observed. Tryptophan has been identified as the main precursor for IAA biosynthesis pathways in bacteria and plants. More than one pathway of IAA synthesis was found to be operated in a single strain of bacteria (Patten and Glick, 1996) and the same thing is expected in plants. IPA was found to be an important intermediate in IAA synthesis in bacteria and a crucial role for this intermediate has been recently confirmed in plants. IAM was also proved functional in bacteria and several lines of evidence for its role in IAA synthesis were found in plants. Differences in IAM reactions between bacterial and plant pathways are also expected.

Because auxin is almost implicated in every step in plant development, enzymes involved in IAA synthesis were expected to be conserved across the plant kingdom. Based on this hypothesis it was expected to find a conserved clade for IAM- hydrolase enzyme proposed to have a role in IAM pathway of IAA synthesis from all studied plant species. Abu-Zaitoon et al. (2012) quantified the free level of IAA in developing rice grains at 1, 4, 7, 10, 14, and 21 DAF using liquid chromatography electrospray ionization / mass spectrometry (LC-ESI-MS). The amount of IAA was found to increase from below the detection limit at 1 DAF to 1.5 µg/gfw at 21 DAF. Rice kernels were therefore used as an experimental material and 1,7,21 DAF were selected to detect the expression profile of AMI.

The major aim of the research described in this work was to discuss in the light of phylogenetic analysis whether the AMI enzyme is conserved across the plant kingdom and therefore its potential importance as an important enzyme in IAA synthesis. Additionally, the involvement of the indole-3-acetamide pathway in IAA production in plants and rice, in particular, will be discussed based on the expression profile of the conserved AMI gene as well as the recent available publications.

MATERIALS AND METHODS

Bioinformatics

Bioinformatics was used to investigate the phylogenetic relationships of AMI enzymes proposed to be involved in the IAM pathway of IAA production. IAM-hydrolase from *Arabidopsis* was used as query sequences. Homologues of the targeted proteins from diverse taxonomic groups of moss, non-seed vascular plants, monocots, and dicots were identified. Constructed phylogenetic tree for IAM-hydrolyase enzymes were then investigated to identify a clade, if any, that are conserved across the plant kingdom.

Homologues of AMI1 were identified following BlastP searches (Altschul et al., 1997) against proteomes of *Arabidopsis thaliana*, *Physcomitrella patens*, *Selaginella moellendorffii*, *Oryza sativa* subsp. *Japonica*, *Zea mays*, *Sorghum bicolor*, *Populus trichocarpa*, *Vitis vinifera*, *Glycine max*, *Fragaria vesca*, *Medicago truncatula*, *Ricinus communis*, *Theobroma cacao*, *Carica papaya*, *Nicotiana tabacum*, *Hordeum vulgare* subsp. *Vulgare*, *Brachypodium distachyon*, and *Picea sitchensis* (Plaza 2.5; http://bioinformatics.psb.ugent.be/plaza/ (Bel et al., 2012; Proost et al., 2009), as well as *Solanum lycopersicum* (The Sol Genomics Network (SGN; http://solgenomics.net/) (Bombarely, et al., 2011). The GenBank database (Non-redundant protein sequences) was screened to search for sequences from other plants. Phylogeny.fr free web service was used to construct and analyze phylogenetic relationships between AMI sequences (Dereeper et al., 2010; Dereeper et al., 2008).

Alignment of amino acids rather than nucleotide sequences was used in this study because it is easier to find unauthentic alignment of DNA sequence which consists of only 4 nucleotides comparing to 20 different amino acids for protein sequences. DNA mutates quickly as a 24% of single base changes produce the same amino acid.

Sequences were aligned with MUSCLE (v3.7 with default settings) configured for highest accuracy. Edgar (2004) reported that MUSCLE can achieve both better average accuracy and better speed than CLUSTALW or T-Coffee, depending on the chosen options. Default parameters were claimed to give the best average accuracy.

Distance-matrix (Fitch, 1981), maximum parsimony (Fitch, 1971), and maximum likelihood (Felsenstein, 1981) are the three major methods used to construct phylogenetic trees. Maximum likelihood method implemented in the PhyML program (v3.0 aLRT) (Guindon and Gascuel, 2003) was used to reconstruct the tree. Due to simplicity as well as accuracy and speed, PhyML has been widely used (>2500 citations in ISI Web of Science) (Guindon et al., 2010). Approximate Likelihood-Ratio Test (aLRT) rather than Bootstrapping procedure was used to statistically test branches support. SH-like algorithm was used to estimate the confidence of the aLRT statistics. Default substitution model was used.

Table 1. Forward and reverse primer sequences used to detect the expression of the rice AMI as well as the reference rice genes (act1, and ubc) in developing rice grains.

Gene	Name	Forward Primer (5`⟶3`)	Reverse Primer(5`⟶3`)	Product size (bp)
Os04g02780	ami	CATCAAGGACATCTTCGACATC	ATCTCGTCCATGATGGTGGT	156
Os05g01600	act1	CTTCATAGGAATGGAAGCTGCGGGTA	CGACCACCTTGATCTTCATGCTGCTA	196
Os02g42314	ubc	CCGTTTGTAGAGCCATAATTGCA	AGGTTGCCTGAGTCACAGTTAAGTG	76

MUSCLE (Edgar, 2004) was initially used to perform multiple sequence alignments. The fractional identity between each pair of sequences is computed using *k*-mer counting. A tree is then constructed from the triangular distance matrix computed from the pair-wise similarities using UPGMA and a root is identified. The resulting tree is improved by building a new progressive alignment and a new tree is constructed by computing a Kimura distance matrix.

SH-like is an algorithm used to estimate the confidence of the aLRT statistics. This method of branch test is considered to be a very fast, alternative and complement to the standard bootstrap analysis. An appropriate array is stored from simply drawn and summed values. Moreover, the nonparametric SH-like support is closely related to P values of statistical tests. Experiments with simulated data indicate that the new SH-like interpretation of the aLRT statistic should be preferred to the parametric chi-square–based interpretation due to unavoidable simplifications of substitution models when analyzing real data. As the standard bootstrap, aLRT with SH-like interpretation is conservative (Guindon et al., 2010).

Plant materials

Rice plants, Oryza sativa (c.v. Jarrah) were grown in a glasshouse in flooded soil-filled plastic pots. Pots were filled with black soil containing Aquasol fertilizers (N: P: K 23:4:18). Temperature inside the green house was 30°C during the day and 18°C during the night. Each branch of rice plants was marked when the top half of the panicle had reached anthesis. Superior caryopses were harvested 1, 7, and 21 DAF. Microfuge tubes containing 60 to 70 mg of rice seed (10, 3, and 2 seeds from 1, 7, and 21 day samples respectively) were then weighed and frozen in liquid nitrogen for total RNA extraction. Samples not used immediately were stored in a freezer at -70°C for later use.

RNA Isolation

Total RNA from rice grains was extracted following the instructions for RNeasy® Mini Kit (Qiagen, Cat. No. 74903). The absorbance of extracted RNA was measured at 260 nm (A 260). The concentration of RNA in µg/mL was calculated as follows: Concentration= 40* A 260* Dilution Factor (QIAGEN, 2002). A nano-drop spectrophotometer (NanoDrop® ND-1000 Spectro-photometer, NanoDrop Technologies, Inc., USA) was also used to measure RNA concentration, A260/A280 and A260/A230 ratios. RNA extracts with high purity, A260/A280 and A260/A230 > 1.8 and 2 respectively, were used in all experiments (QIAGEN manual). RNA quality was checked by electrophoresis in 1.2% agarose run at 100V for 45 min after heating at 80 °C for 10 min and cooling on ice (Nolan et al., 2006).

Polymerase chain reaction

The program, primer3 (Rozen, 1998) was used to design primers for the cDNAs of all targeted genes. Primer sequences are listed in Table 1. Gene-specific primers were designed so that either the left or right primer was complementary to an exon-exon boundary to exclude amplification of genomic DNA. Primers were dissolved in fresh sterile milli-Q water to prepare 50 µM stock solution; 10 µM working solutions were prepared and frozen at -20 °C.

The reverse transcriptase polymerase chain reaction with gene-specific primers was run according to the instructions of the manufacturer (QIAGEN®, OneStep RT-PCR Kit, Lot No. 133206209). Thermal cycler (PTC-100 TM Programmable Thermal Controller, MJ Research, Inc., USA) conditions are listed in Table 1. The same amount of RNA extracts (1.2 µg) was used to perform all RT-PCR reactions. 3 µL each of 10 µM primer solution was added to a final concentration of 0.6 µM. RT-PCR was attempted using 30 cycles. To estimate the size of each RT-PCR product, BenchTop 100 bp DNA Ladder was used (Promega). The intensity of the 500 band is three times higher than that of the other equal intensity bands and as a result can be used as a reference band.

Purification and sequencing

For PCR, the amplification protocol was the same as indicated for RT-PCR but with 35 cycles and without the reverse transcriptase reaction. cDNA products were purified according to the Wizard SV Gel and PCR Clean-Up System (Promega, Cat. # A9281). Concentration of cDNA samples was calculated according to the following equation Concentration= 50* A 260* Dilution Factor (Sambrook and Russell, 2001). Full-length cDNA of all genes were sequenced by Prince Alfred Molecular Analysis Centre, Sydney University.

RESULTS AND DISCUSSION

Phylogenetic relationships of IAM-hydrolase from the available sequenced plant genomes were investigated. *Arabidopsis* AMI1 (AtAMI1) was used as a query sequence to search for all putative AMI in plants. As a result of BlastP search using the available plant proteomic data bases, 154 putative AMI sequences were found. The number of sequences ranges from 1 for *P. sitchensis* to 21 for *P. trichocarpa*. To simplify the phylogenetic tree, sequences from the same species that are much more similar to each other rather than to sequences from other plants were omitted. Therefore, the number of sequences was reduced to 138.

The phylogenetic tree for the 138 IAM hydrolase sequences (figure not shown due to the large number of sequences) showed that all sequences are classified into two major branches. The majority of plant sequences are found in the first major branch that is supported at 88% by statistical analysis. For example *O. sativa*, *Z. mays*

and *S. bicolor* have 9, 6 and 7 sequences, respectively in this branch. The *Arabidopsis* sequence (At5g64440) that was not found to hydrolyze indole-3-acetamide detectably is classified in the first branch suggesting that sequences in this branch may not be involved in IAA synthesis. At5g64440, a fatty acid amide hydrolase (Shrestha et al., 2003), has the ability to convert *N*-acylethanolamine to ethanolamine and the corresponding free fatty acids. Large amount of ester IAA for plants including rice may explain the large number of putative amidases in this branch. Enzyme kinetics and substrate specificity of the other two *Arabidopsis* sequences (At5G07360, At3G25660) in this clade needs to be worked out. However, these two sequences have the essential residues of the supposed amidase activity.

To get a more precise picture, phylogenetic tree for all sequences in the second major branch was reconstructed at high stringent selection (Figure 2). Unlike the first major branch, all studied plants were found to have representative sequences in this branch. The second major branch that is supported at 100% by statistical analysis is divided into two minor branches. The first minor one, supported at 94% by statistical analysis, contains the *Arabidopsis* sequence (At5G09420) that does not found to have amidase activity suggesting that representatives of this branch may not be involved in IAA synthesis. At5G09420 is localized in the outer mitochondrial membrane in complex with a protein translocase (Chew et al., 2004).

The second minor branch, supported at 94% by statistical analysis, is also divided into two clades. The well characterized *Arabidopsis* amidase 1(AMI1) and *N. tabacum* 1 (NtAMI) as well as the only two sequences of *H. vulgare* are found in the first clade that was supported at 100% by statistical analysis. The only sequence of *P. sitchensis* is also found in this clade. No any sequences for *P. patens* and *S. moellendorffii* were identified in this clade. Unlike other *Arabidopsis* putative amidases that was not found to be involved in IAA synthesis and found in mitochondria or chloroplast, the intracellular location of the AtAMI1 was identified in the cytoplasm where IAA synthesis occurs (Pollmann et al., 2006). All sequences except Pt13G02310 in this clade contains the essential residues of the supposed amidase catalytic triad (Lys-*cis*Ser-Ser) (Shin et al., 2003) (Figure 3). The first clade is conserved and expected to have a role in IAA synthesis or other primary role in plant growth and development. Characterization of amidases in this clade from different plants especially those of agricultural importance that are generally used in IAA studies; i.e rice, maize, and tomato, will gather pieces of knowledge to form a complete picture of IAM in plants.

The second clade, supported at 74% by statistical analysis, contains the *Arabidopsis* sequence (At3G17970) which is not expected to have amidase activity. At3G17970, a part of the preprotein translocon of the outer envelope of chloroplasts (Toc64-III), shows some structural differences from AtMI1. Toc64-III contains tetratricopeptide repeat motifs in its C-terminal domain that are found in the ancient moss *P. patens* (Hofmann and Theg, 2003) but not the essential residues of the supposed amidase catalytic triad.

IAM hydrolase was partially purified from rice cells and reported to hydrolyze IAA ethyl ester as well as IAM and its homologue 1-naphthalene acetamide but not indole-3-acetonitrile (Arai et al., 2004). Although the molecular mass of the enzyme was very similar to that of the *Agrobacterium tumefaciens*, a significant role for this enzyme in IAA synthesis was excluded due to the considerably high km value. AMI generally has dual functions as amidase and esterase. Together with the observation that rice ears contain a large amount of esterified IAA at the stage of anthesis (Kobayashi et al., 1989). Arai et al. (2004) suggested that this enzyme may serve to control IAA accumulation via the hydrolysis of IAA. In this study we reported that rice have 15 putative IAM hydrolase enzymes, one of them (Os04G02780) is in the conserved clade. As a preliminary result, a transcript for Os04G02780 was detected in rice grain at 1, 7 and 21 days after anthesis where large amount of IAA is accumulated during this period of growth (Figure 4). Therefore, it is expected for Os04G02780 to have a significant role in IAA synthesis via the IAM intermediate.

Pollmann et al. (2009) isolated IAA synthase, an enzyme complex that can convert Trp to IAA, from 14 different plant species. They suggested that IAM is an intermediate in IAA synthesis by this complex due to the finding that IAM interferes with the conversion of 1mM $[^2H]_5$-Trp to $[^2H]_5$- IAA. The reduction in IAA conversion was reported to b 66% and 80% in case of 1 mM and 0.1 mM IAM respectively. IAA synthase was also reported to convert labeled IAM to IAA and increasing concentrations of Trp interfered with this conversion. The amount of IAM in *Arabidopsis* seedlings was found at levels similar to that of free IAA (Pollmann et al., 2002). Based on the higher conversion rate of L-Trp and $[^2H]_5$-IAM to IAA comparing to excess L-Trp, Pollmann et al. (2009) concluded that the formation of IAM rather than the conversion of IAM to IAA is the rate limiting step in IAM pathway of IAA synthesis.

The physiological importance of the IAM pathway of IAA synthesis came from the observation that transgenic BY-2 cells overexpressing *N. tabacum* AMI1 could grow in medium containing low concentrations of IAM, whereas suppression of the *NtAMI1* gene by RNA interference caused severe growth inhibition in medium containing IAM. These results clearly show that the *NtAMI1* gene is expressed in plant cells and is required for the conversion of IAM to the auxin IAA that involved in plant cell division (Nemoto et al., 2009).

The same scenario as IPA pathway in bacteria and plants, the possibility for the direct conversion of Trp to IAM and therefore the 2-step IAM pathway of IAA synthesis in plant as bacteria is not ruled out. Generating

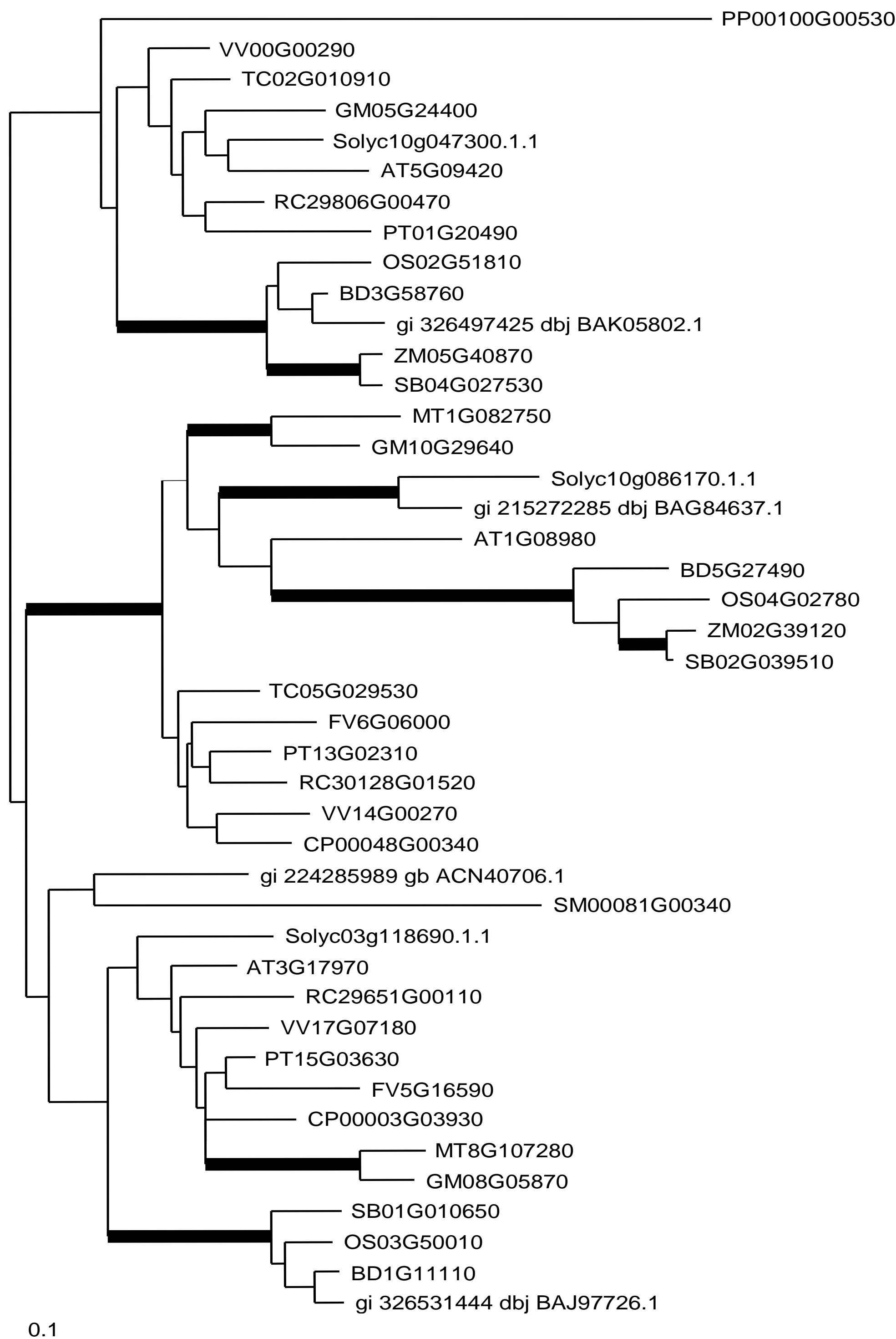

Figure 2. The tree was constructed using MUSCLE with default settings (v3.7) to align sequences (Edgar, 2004). Maximum likelihood method implemented in the PhyML program (v3.0 aLRT) (Guindon and Gascuel, 2003) was used to reconstruct the tree. Reliability for internal branch was assessed using the aLRT test (SH-Like). TreeDyn (v198.3) (Chevenet et al., 2006). Bold line branches indicate that they have 95% statistical support or more. The phylogenetic tree was edited and represented using TreeView (Page, 1996). The tree analysis was performed on the Phylogeny.fr platform (http://www.phylogeny.fr/version2_cgi/index.cgi). The scale bar represents 0.1 substitutions per amino acid. A 43 taxa with average sequence lengths of 524 amino acids (between 352 and 637) from the second branch of the constructed tree in figure 1. This tree was constructed at high stringent selection.

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                                        ★
RC30128G01520                  -LHSLTFAVKDIFDVDGYVTGFGNPDWARTHSAATATAPAVLAVLRGGAT 147
PT13G02310                     -LHGLTFAVKDIFDVEGYVTGFGNPDWARTHSAATSTAPAVLAVLRGGAT 75
TC05G029530                    -LSGLTFAVKDIFDIEGYVTGFGNPDWARTHSAATTTAPAVMDLLKGGAT 75
FV6G06000                      -LSGLTFAVKDIFDVAGYVTGFGNPDWARTHPGAESTAPSISTILRGGAT 75
VV14G00270                     -LNGLTFAVKDIFDVDGYVTGFGNPDWARTHQAAMLTAPSVLAVLKGGAT 75
CP00048G00340                  -LTGLTFAVKDIFDVDGYVTGFGNPDWARTHSAATSTASAVLAVLEAGAA 75
GM10G29640                     -LKSLTFAVKEIFDTEGYVTGFGNPDWARTHPVATSTAPTALALLRAGAT 105
MT1G082750                     -LNSLTFAVKDIFDVKGYVAGFGNPDWARTHQAATSTAPTVLAILSAGAT 75
AT1G08980                      SLQGLTFAIKDIFDVEGRVTGFGNPDWLRTHSAATSTAPVVSSLLEAGAT 76
gi|215272285|dbj|BAG84637.1|   PLNGLTFAVKDIFDVEGHITGFGNPDWAKTHSAATSTATTVLTLLKAGAT 75
Solyc10g086170.1.1             ELNGLTFAVKDIFDLEGQITGFGNPDWGKTHSAAICTAPTVLSLLKSGAT 76
SB02G039510                    PLHGLTFAIKDIFDISGRVTGFGNPDWARTHAPAGATSPVVLATLAAGAI 75
ZM02G39120                     PLHGLTFAIKDIFDIGGRVTGFGNPDWARTHAPAGATSPVVLAALAAGAI 80
OS04G02780                     PLHGLTFAIKDIFDIAGRVTGFGNPDWARTHAPAAATSPVVLAALAAGAT 78
BD5G27490                      PLHGLTFAIKDIFDIAGRVTGFGNPDWSRTHGPAAATAPAVLALLAAGAA 79
                                * .****:*:***  * ::******* :**  *  *:.     * .**

                                                                     ★
RC30128G01520                  CVGKVIMDEMAYSINGENAHYGTPTNPSAPDRVPGGSSSGSAVAVGAKLV 197
PT13G02310                     CVGKTIMDEMAY-------------------------------------- 87
TC05G029530                    CLGKTVMDEMAYCMYGVNKHYGTPTNPCAPDRVPGGSSSGSAVAVGAMLV 125
FV6G06000                      CIGKTVMDEMAYSINGENKHYGTPVNPCAPDRVPGGSSSGSAVVVGADLA 125
VV14G00270                     CVGKTVMDEMAYSINGENKHYGTPTNPQAPDRVPGGSSSGSAVAVGAMLV 125
CP00048G00340                  CVGKTVMDEMAYSINGENKHYGTPTNPCARDRVPGGSSSGSAVAVGAKLV 125
GM10G29640                     CVGKTVMDEMAYSINGENIHYGTPRNPCAPDRVPGGSSSGSAVAVGAKLV 155
MT1G082750                     CVGKTVMDEMAYSINGENIHYGTPRNPCAEDRVPGGSSSGSAVAVGAKLV 125
AT1G08980                      ALGITIMDEMAYSINGENAHYGTPRNPIAFDRVPGGSSSGSAVAVAARLV 126
gi|215272285|dbj|BAG84637.1|   CIAKTVMDEMAYSINGENVHYGTPVNPVSPDRVPGGSSSGSAVAVGAKLV 125
Solyc10g086170.1.1             CIGITVMDEMAYSINGENFHYGTPVNPVAPDRVPGGSSSGSAVAVGAKIV 126
SB02G039510                    SIGKTVMDEMAYSINGENAHYGTPTNPCAPDRVPGGSSSGSAVAVGAKLV 125
ZM02G39120                     SIGKTVMDEMAYSINGENAHYGTPTNPCAPDRVPGGSSSGSAVAVAAKLV 130
OS04G02780                     SLGTTIMDEMAYSINGENTHYGTPTNPCAPGRVPGGSSSGSAVAVAANLV 128
BD5G27490                      AVGRTVMDEMAYSINGENAHYGTPANPCAPDRVPGGSSSGSAVAVAASLA 129
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RC30128G01520                  DFSLGSDTGGSVRVPASYCGILGFRPSHGVVSVSGVIPMAQSFDTVGWFS 247
PT13G02310                     ----------SVRVPASYCGILGFRPSYDAVPSAGVVPLAQSFDTVGWFA 127
TC05G029530                    DFSLGTDTGASVRVPASYCGILGFRPSLGAISTVGVVPMSQSFDTVGWFA 175
FV6G06000                      DFSLGTDTGGSVRVPASYCGIFGFRPSHGAISTSGVTPMAQSFDTVGWFA 175
VV14G00270                     DFSLGTDTGGSVRVPASYCGIFGIRPSHGVVSTTGVIPMAQSFDTVGWFA 175
CP00048G00340                  DFSLGTDTGGSVRIPASYCGIFGFRPSHNAVSTAGVIPMAQSFDTVGWFA 175
GM10G29640                     DFSLGTDTGGSVRVPASYCGIFGFRPSHGAVSESGVIPMSQSFDTVGWFA 205
MT1G082750                     DFSLGTDTGGSVRVPASYCGIFGFRPSHGRISKSGVVPMAQSFDTVGWFA 175
AT1G08980                      DFSIGTDTGGSVRVPASYCGIFGFRPSHGAVSTVGLTPMAQSFDTVGWFA 176
gi|215272285|dbj|BAG84637.1|   DFSLGTDTGGSVRVPASYCGIYGIRPSHGVVSTVGVTPMAQSFDTVGWFA 175
Solyc10g086170.1.1             DFALGTDTGGSVRVPASYCGIYGIRPSHGVVSVDGVIPMAQSFDTVGWFA 176
SB02G039510                    DFALGTDTGGSVRVPAAYCGIFGLRPSHGLVSTENVIPMSQMFDTVGWFA 175
ZM02G39120                     DFALGTDTGGSVRVPAAYCGIFGLRPSHGLVSTENVIPMSQMFDTVGWFA 180
OS04G02780                     DFSLGTDTGGSVRVPAAYCGIFGLRPSHGLVSAENVIPMAQMFDTVGWFS 178
BD5G27490                      DFSLGTDTGGSVRVPAAYCGIFGLRPSHGLVSTENVIPMAQMFDTVGWFA 179
                                         ***:**:**** *:*** . :.  .: *::* *******:
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Figure 3. Triad of the amidase activity domain in all sequences of the clade represented by asterisk. Locus number of each sequence is shown on the left side whereas the perspective amino acid position is shown on the right side.

a mutant with a decreased level of IAM will help to find out the enzyme that catalyses the rate limiting step of this pathway. Making a microarray screens for the expression level of plant genes before and after silencing IAM

Figure 4. RT-PCR analysis of the rice *AMI* gene. The rice act1 and ubc were used as reference genes. Reactions used 1.2 μg of RNA extracted from developing rice grains at 1, 7 and 21 days after anthesis (DAF). RT-PCR conditions are outlined in table 1. 30 cycles of amplification and 54°C annealing temperature were used in this experiment. Other genes appear in this figure is related to other study.

hydrolase may give us some indications about the change in the gene level for the enzyme catalyze the missed step in the IAM pathway.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

The effects of storage conditions on the viability of enteropathogenics bacteria in biobanking of human stools: Cases of *Yersinia enterocolitica*, *Salmonella enterica* Typhimurium and *Vibrio cholerae* O: 1

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Long-terms recoverability of enteropathogens is necessary for future epidemiological studies to screen stool samples when conditions do not permit immediate processing. The aim of this study was to determine the viability and the recoverability of three enteropathogens bacteria (*Yersinia enterocolitica*, *Vibrio cholerae* O: 1 and *Salmonella enterica* Typhimurium) artificially contaminated at 10^7 CFU/ml in human stool samples after our storage conditions. Preservations media used were saline, phosphate buffered saline (PBS) and Modified PBS without glycerol and containing 10 and 20% glycerol at three temperatures (4, -20 and -80°C). The viabilities and the recoverabilities of these strains were determined (weekly and monthly) respectively by plating onto tryptic soy agar and detection from artificial inoculated stools samples onto specifics agars. Bacteria populations decreased by 1-5 $\log_{10}$ CFU/ml depending on the strains tested, on the preservation media, on the glycerol concentration and the storage condition. The greatest population decrease was observed in the first week of storage at +4°C and freezing at -20°C and a slow decline in survival occurred thereafter. No organisms were recovered after one month from samples at these temperatures. When samples were subjected to storage at -80°C, all enteropathogenics bacterial were recovered after nine months storage.

Keys words: Biobanking, enteropathogenics bacteria, storage conditions, preservation media, Côte d'Ivoire.

INTRODUCTION

In developing countries, diarrheal diseases are a major public health problem that particularly affects children.

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Due to the very high infant mortality in the world more often in African countries (Boschi-Pinto et al., 2008), it is necessary to acquire knowledge on the etiological and epidemiological characteristics of diarrhea (Randremanana et al., 2012) and to have a clear view of the situation in order to design specific therapeutic measures and control strategies.

However, the ability to detect an etiologic agent is lack in many parts in remote regions of the world, especially where diarrheal disease is the most common and where most infant mortality occurred (Petti et al., 2006). Transportation of clinical specimens to specialized centers and long-term recoverability of enteropathogens are also a major problem in bacterial diagnosis, particulary when the conditions do not permit the immediate processing (Acha et al., 2005).

The long-term storage of stools samples or rectal swabs is therefore useful to carry out studies in several research field. That can also provide an opportunity to reassess later old specimens to screener emerging or remerging pathogens. Methods for the long-term storage of bacterial strains were well established, and several methods are available. Previous studies on the effect of storage conditions are laid mainly on the survival of bacteria (Yamamoto and Harris, 2001; Gorman and Adley, 2004; Ternent et al., 2004), but very few experimental studies have been conducted on the storage of biological polymicrobial specimens especially stools samples.

Freezing is the preferred method for storage of specimens of faeces but the problem with this method is the harm caused by the freezing and thawing according to several authors (Freeman and Wilcox, 2003; Acha et al., 2005).

In Côte d'Ivoire with limited financial resources, the freezing at -20°C is the most storage widely used in many health centers. Also the use of non expensive preservation media is suitable for storage of stools samples.

The aim this study was to determine a pretreatment method and storage conditions of stools samples effects on the viability of enteropathogenics bacterial strains during their biobanking.

MATERIALS AND METHODS

Sampling and bacteria strains

This study was carried out from February to November 2013 at the Department of biological ressources (Biobank) of the Institut Pasteur in Côte d'Ivoire. It consisted of four human stools freshly issued and three reference strains of *Yersinia enterocolitica* IP383, *Vibrio cholerae* O:1 *Salmonella enterica* Typhimurium from bio-collection of the Institut Pasteur in Côte d'Ivoire and the Pasteur Institute in Paris.

The fecal samples included in this study were free for these enteric bacteria and were collected from healthy people.

Pretreatment of fecal samples

Thiel and Blaut (2005) described the pretreatment method with some modifications. Briefly, 2 g of fresh stools were transferred into a sterile falcon tube (15 ml) and diluted in 10 ml of different preservation media which was to be tested. These preservation media were: Phosphate Buffered Saline (containing 150 mM NaCl, 10 mM NaH_2PO_4, 20 mM NaH_2PO_4, pH 7.4), the saline (0.9% NaCl), and modified PBS (0.1% peptone, 0.25% yeast extract).

These preservation media were prepared without glycerol or with glycerol at 10 and 20% in final concentration. The samples were homogenized by adding ten sterile glass beads (3 mm of diameter) and stirring for 3 min using vortex mixer (Sterilin ® STUART LTD). Thereafter, the fecal samples were centrifuged at 3000 rpm for 5 min to remove the glass beads and larger solid particles (debris) contained in the stool.

Quantification of bacterial

From a pure culture of reference strains of 18 to 24 h onto non-selective agar, a bacterial suspension was prepared in 5 ml of suspension medium (Biomerieux ®) at 0.5 Mac-Farhland equivalent to 10^8 CFU/ml using a DENSIMAT (VITEK, Biomerieux ®). The fecal samples were pretreated and the control media were contaminated with bacteria suspensions in a 1: 10 proportion (1 ml of bacterial suspension + 9 ml of stool). This gave an initial concentration of 10^7 CFU/ml in the samples. Several aliquots (1 ml) were then made and packaged in cryovials and Eppendorf tubes. Each stool sample and his control were stored at different storage temperatures (+4, -20 and -80°C) for 9 months.

Viability tests

The viability test was consisted of a simultaneous detection and counting of bacteria, respectively, in contaminated storage media and stool samples.

Before any weekly and monthly analysis, all samples were placed at room temperature for thawing. The numbering was performed after a serial of decimal dilution, and 0.1 ml was spread onto tryptic soy agar TSA (Biorad). For bacterial detection a loopful of test contaminated stool samples was streaked on culture medium appropriate to the strain to be recovered:TCBS agar (Scharlau) for *Vibrio cholerae*, Hecktoen agar (Lyofilchem) for *Salmonella enterica* and CIN agar (Cefsulodin Irgasan Novobiocin, *Yersinia* Selective Agar Base and *Yersinia* Supplement, Liofilchem) for *Yersinia enterocolitica*.

All agar plates were incubated at 37°C except CIN agar which was incubated at 30°C. The number of colony forming unit (CFU) of each preservation medium was determined by the following formula according the French Nor (AFNOR, 2002):

$$N(CFU/ml) = \frac{\sum C}{V(n_1 + 0{,}1 n_2)d}$$

Where, ΣC is the The total of all colonies counted on the agar plate in two serial decimal dilutions; V is the volume of the inoculum applied on agar plate; n1 is the the number of plate retained for the first considered dilution; n2 is the the number of plate retained for the second considered dilution and d is the the first dilution considered

Statistical analysis

To compare the results of all assays, statistical analysis was carried

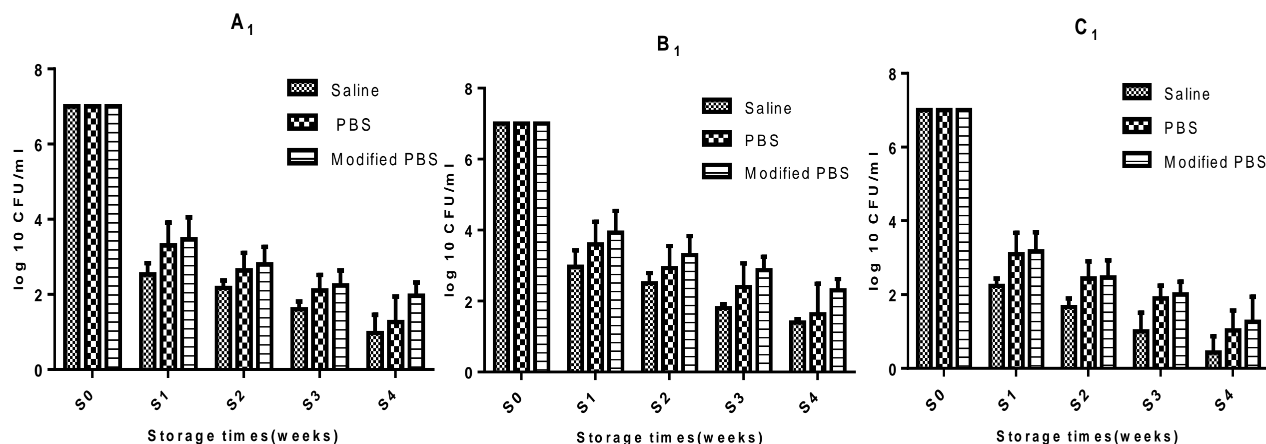

Figure 1. Recovery of enteropathogenics bacteria stored in continuous cyclic frozen without thawing in the preservation media at -20°C. **A_1,** *Yersinia enterocolitica* IP383. **B_1,** *Salmonella enterica* Typhimitium. **C_1:** *Vibrio cholera* O:1.

out using a GraphPad Prism version 5 software. The values reported define the boundaries of an interval with 95% certainty. The results were only considered statistically different at $P < 0.05$.

RESULTS AND DISCUSSION

To date, there is no ideal medium that preserves the viability of pathogens and flora without allowing the growth or inhibition of some species (Wasfy et al., 1995). The preservation of pathogen viability in biological specimens is also essential for their recovery. This is especially true for faecal specimens; overgrowth of normal faecal flora can mask enteropathogenics and impair isolation.

In our study, after storage at 4°C, most strains were not viable for more than one week. Exceptionally *Yersinia enterocolitica* was viable and recoverable in all preservation media more than one month. These results are comparable to those of Dan et al. (1989) when a transport medium was not used. The ability of *Y. enterocolitica* to grow at low temperature due to its psychrotrophic character but the other bacteria strains are mesophilic.

In our study, the recoverability and viabibity of enteropathogenics species at -20°C were ambiguous. The directly frozen of stools samples without cryoprotective agent (glycerol) in the preservation media at -20°C was catastrophic for all enteropathogenics bacteria.

Poor results were obtained when stools samples were frozen for one week at -20°C. With the greatest glycerol concentration (20%) *Vibrio cholerae*, *Y. enterocolitica* and *Salmonella enterica* Typhimirium underwent globally 3 to 4,7 $\log_{10}$ CFU/ml population decline after one week storage in the different preservation media (Figure 1). Non-significant result was obtained between all the preservation media. The different bacteria species may vary in their susceptibility to damage by freezing and thawing.

At this low temperature, the pH and biochemical activities of stool microbiota (aerotolerant anaerobes) have been reported to be detrimental to the viabilities of pathogenic organisms in fecal samples (Lennette et al., 1985). All the enteropathogenics species tested were preserved at -80°C for the entire period of study and quantitative recovery was very satisfactory whereas there was a difference between the results obtained with the various preservation media, and between storage with the different glycerol concentration in the preservation media. All preservation media with 20% glycerol gave also better results.

The loss of viability was also strain-dependent ($p<0.05$). In this study, *Salmonella enterica* was better recovered than *Y. enterocolitica* followed by *Vibrio cholerae. V. cholerae* was the most sensitive strains exhibiting 3,8 to 4,9 $\log_{10}$ CFU/ml decrease comparatively to *Yersinia* and *Salmonella* strains whose showed respectively 2,9 to 4 $\log_{10}$ CFU/ml and 2,3 to 2.9 $\log_{10}$ CFU/ml decrease during the study period.

The comparison of bacterial strains sustainability, viability and forever recoverabilty during this storage method in preservation media showed that there was not a difference between saline and PBS and between PBS and modified PBS for all bacterial strains. The difference was observed only between saline and modified PBS. A significant difference ($p<0.05$) was observed at the seventh month storage for *Y. enterocolitica* strain and this difference was extremely significant ($P<0.01$) during the two last months (Figure 2). Right from the fourth month storage, difference was observed for Salmonella and after six (6) months storage, the decrease was similar in the all preservation media. That also has been observed

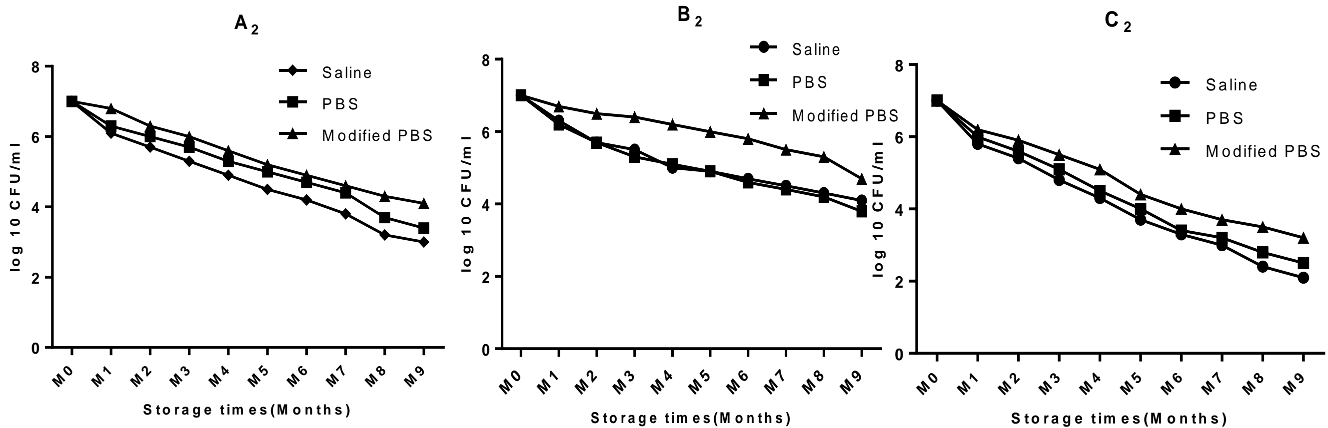

Figure 2. Enteropathogenics bacteria recovered in continuous cyclic frozen in the preservation media with 20% glycerol at -80°C. **A₂,** *Yersinia enterocolitica* IP383. **B₂.** *Salmonella enterica* Typhimitium. **C₂,** *Vibrio cholera* O:1.

for *V. cholerea* strain but from the eighth month storage this decrease was more significant.

This observation is valid for all bacteria studied but strains *of V. cholerae* seems to lose viability quickly, and the long-term cultivability. Conversely, when stool samples were stored at -80°C in buffered media the viability and recoverability of enteropathogenics bacteria were better compared to those obtained without storage medium and physiological saline. The sharpest decline in viability was observed in saline and PBS but better recoverability seems to be obtaining with modified PBS media. This suggestion may disagree with the view that the low nutrient content of Carry Blair and utilization of phosphate as a buffering agent may provide suitable sample viability and control of contaminants that may mask the recovery of true pathogens.

We conclude that most enteropathogens can survive in faecal samples for as long as nine months when stored only at lowest temperature -80°C. These bacteria can also be preserved, at least partially, for the same period, if preservation media are used in conjunction with low temperatures.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Application of molecular markers to find out classificatory determinants of rice (*Oryza sativa* L.)

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The present communication is aimed to find out determinants of molecular marker based classification of rice (*Oryza sativa* L) germplasm using the available data from an experiment conducted for development of molecular fingerprints of diverse varieties of Basmati and non Basmati rice adapted to irrigated and aerobic agro-eco systems. The distance-matrix based dendrogram was developed following UPGMA method that grouped all the 44 varieties agro-eco systems wise. We examined the clustering of Basmati and non- Basmati varieties in detail. The results suggest that the easily distinguishable simple characters viz. plant height, photosensitivity, leaf color, awn's color, awns' number etc., governed by major genes did not contribute significantly towards classification of rice genotypes used. The complex characters such as adaptation to agro-eco systems and rice quality may play vital role in determination of molecular marker based classification of rice genotypes.

Key word: Basmati rice, molecular markers, genetic diversity, agro-eco systems, rice quality.

INTRODUCTION

The traditional varieties of Basmati rice are tall, long duration, photosensitive and contained minimum genetic diversity. Therefore, this crop could not be genetically benefitted even during green revolution era. However, low yielding, disease susceptible traditional varieties of Basmati rice still remained in cultivation due to their high quality that fetch high premium price in the local as well as international market.

Lodging and disease appearance in such basmati rice varieties results into poor productivity and inferior quality (DelaCruz and Khush, 2002) which in turn results into less remuneration to the farmers in high input agriculture (Singh and Gupta, 1985). Furthermore, short duration and input efficient rice varieties of inferior quality like Sarbati resembling with basmati in appearance became a challenge for Basmati trade.

Due to high price difference such varieties are used for blending in export consignments that loses national

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credibility in the global market. Triggering efforts, to promote production and maintaining purity of Basmati rice, Government of India has declared this traditional basmati growing area as Agri Export Zone for Basmati rice (Singh et al., 2011; Singh and Sirohi, 2005). Traditionally used morphological and physico-chemical parameter does not provide more precise techniques for maintenance of purity of the commodity (Nagaraju et al., 2002). Therefore, foolproof instant safeguards are required to be developed to detect adulteration of any type of rice blended in the export consignments

In parallel, reliability and precision of such discriminatory functions will apparently depend upon adequacy of molecular markers used and characters contributing towards estimates of genetic diversity. Once adequate molecular markers and efficient determinants are identified, further studies will be intensified to get information at finer level which could be potent to precisely detect admixture in commercial varieties (Botstein et al., 1980; Vos et al., 1995). Alternatively, it is needed to find out characteristics/determinants contributing to genetic diversity (Singh, 2010) which is the basis of classification/clustering of genotypes.

So, the present study was undertaken with the objective to fingerprint/characterize different varieties of Basmati and non-Basmati rice adapted to different agro-ecological systems by using SSR and its alternative approach inter-SSR assays (Zietkiewicz et al., 1994, Singh et al., 2000a, 2000b) and to examine the results in order to work out efficient determinants for molecular marker based classification.

MATERIALS AND METHODS

Plant materials and molecular markers assay

Forty four varieties of Basmati and non-Basmati rice along with their variety wise place of development/ origin and visibly distinguishable characteristics are listed in Table 1. The genomic DNA of 44 rice varieties was isolated using CTAB method (Moller et al., 1992). 10 ISSR and 28 SSR molecular markers (Table 4) were used for genetic analysis. The ISSR-PCR technique (Zietkiewicz et al., 1994) was used to enhance the speed of sensitivity of detection of molecular markers. The 5', 3' anchored ISSR, and SSR primers synthesized by Bangalore Genei were used. Each reaction mixture (20 ul) that used ISSR and SSR primers for amplification consisted of 10x assay (10 mM Tris HCl, pH 8.0, 50 mM KCl with 3.0 mM $MgCl_2$): 1 U of *Red Taq* DNA polymerase, 1.0 mM each of dATP, dTTP, dCTP and dGTP, 10 µm of primer (Bangalore Genei) and approximately 25 ng of genomic DNA for Inter-SSR and SSR, respectively.

The PCR amplification conditions for ISSR analysis were as follows: initial extended step of denaturation at 94°C for 4 min, followed by 45 cycles of denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, elongation at 72°C for 2 min, followed by extension step at 72°C for 7 min. For SSR analysis, the number of cycles was 35 and annealing temperature was 55°C, respectively. PCR products were mixed with 5 µl of gel loading dye (1x buffer, Bromophenol blue, 0.1%; xylene cyanol 0.1%; and glycerol in water, 50%). The amplification products were electrophoresed on 1.5 to 3% SFR agrose gel at 3 to 5 V/cm in 1x TAE buffer. Genomic DNA was quantified by UV absorbance at 260 nm and 280 nm, using UV Bio-Rad spectrophotometer.

Data analysis and detection of genetic diversity

Forty-four rice varieties were used to estimate genetic diversity. Polymorphic products from ISSR-PCR and SSR-PCR assays were calculated qualitatively for presence (1) or absence (0). The proportion of bands that have been shared between any of the two varieties averaged over loci ISSR and SSR primers were used as the measure of similarity. Genetic diversity was calculated using the following formula (Botstein et al., 1980):

$$\mathrm{PIC}_i = 1 - \sum_{j=1}^{n} P_{ij}^2$$

Pij is the frequency of the *j*th allele for marker *i* and the summation extends over *n* alleles. The calculation was based on the number of bands in ISSR and the number of bands per primer for SSR. Cluster pattern was based on distance matrices by using the Unweighted pair group method analysis (UPGMA) program in WINBOOT software (Yap and Nelson, 1996). The diversity or similarities between varieties were given in the form of dendrogram (Figure 1). For the sake of brevity, dendrogram for combined ISSR-SSR assays is given in Figure 1.

Evaluation of grain and quality

For determination of the length breadth (L/B) ratio, kernel length, breadth of milled and cooked rice, aroma and alkali spreading value (ASV) (Dela Cruz and Khush, 2000), 20 fully developed wholesome milled rice kernels were measured. The kernel elongation ratio (ER) was estimated as ratio of length of the cooked kernels to that of uncooked kernel.

Determination of aroma was based on panel reports. Traditional Basmati varieties Taroari Basmati and evolved basmati variety Pusa Basmati-1 were used as standards. The strength of the aroma was scored sample wise (Sood and Siddiq, 1978) as Strongly Scented (SS) and Mildly Scented (MS). ASV was estimated based on visual rating of starchy endosperm (Little et al., 1978). Twenty rice kernels were incubated in 1.7% potassium hydroxide solution in a Petri dish for 23 h at 30°C.

In addition, evaluation of quality was also made by a panel of experts for overall acceptability ratings on account of its acceptability for appearance, cohesiveness, tenderness on touching and chewing and, aroma, taste, elongation and flaky texture on cooking (Anonymous, 2007).

RESULTS AND DISCUSSION

The molecular marker based available techniques are used to estimate genetic diversity that may correspond to contribution of different characteristics in the category-zation of germplasm. Development of molecular markers was cost effective by using RFLPs, AFLP, microsatellites, inter-SSR and RAPDs. Primary applications of molecular markers are: molecular characterization, classification of germplasm and mapping of the genes. Therefore, these markers are available in public domain. A variety of SSR markers, the most robust molecular markers is potent enough to tag the genes with small differences of 2 to 3 nucleotides for development of genetic maps and

Table 1. Rice varieties (*Oryza sativa*) consisted of Basmati, scented non-Basmati, non-scented fine and coarse rice groups adapted to Irrigated and aerobic agro-eco systems across Asian countries.

Variety Name	Origin	Characteristic features
Pusa -2511	IARI, New Delhi, India	Irri, dwarf, medium duration, super fine, disease, LS, scented
Type-3	Nagina, UP, India	Irri, tall, long duration, photosensitive, disease, TB
Ranbir Basmati	J & K, India	Irri, tall, long duration, photosensitive, disease , TB
Taroari Basmati	CCS HAU, Kaul, India	Irri, tall, long duration, photosensitive, disease, TB
Haryana Basmati-1	CCS HAU, Kabul, India	Irri, semi dwarf, super fine, disease, L S, Basmati, EB
Pusa Basmati-1	IARI, New Delhi, India	Irri, semi dwarf, long duration, large awns, disease, EB
CSR-30	CSSRI, Karnal, India	Irri, tall, long duration, photosensitive, disease, EB
Basmati-370	PAU, Ludhiana, India	Irri, tall, long duration, photosensitive, disease, TB
Pusa-1121	IARI, New Delhi, India	Irri, semi dwarf, medium duration, disease, EB
Vallabh Basmati-21	SVPUAT, Meerut, India	Irri and aerobic, semi dwarf, medium duration, EB
MAUB-57	SVPUAT, Meerut, India	Irri, semi dwarf, long duration, photosensitive, EB
Super Basmati	PAU, Ludhiana, India	Irri, tall, long duration, photo sensitive, disease, EB
N-22	Nagina, UP, India	Irri, semi dwarf, drought resistant, disease, non -scented,
Govind	GBPUAT, UK, India	Irri, dwarf, non-scented, short duration, coarse, L S, non-scented
NDR-118	NDAUT, UP, India	Irri, dwarf, medium duration, coarse, L S, non- scented
HKR-1	CCS HAU, Kaul, India	Irri, semi dwarf, medium duration, disease, LS, scented
Sarbati-B	Farmers' variety UP, India	Irri, semi dwarf, fine, short duration, lodging, disease, non-scented
Sarbati-A	Farmers' variety UP	Irri, tall, fine, short duration, lodging, disease, non- scented
Pusa Sungandha-2	IARI, New Delhi, India	Irri, fine, semi dwarf, medium duration, L S, scented
Pant Dhan-10	GBPUAT, UK, India	Irri, semi dwarf, medium duration, L S, non- scented
Pant Dhan-12	GBPUAT, UK, India	Irri, semi dwarf, medium duration, L S, non- scented
Vallabh Bangani	SVPUAT, Meerut, India	Irri, dwarf, medium duration, violet color foliage, non-scented
Sathi-Safed	Farmers' Variety UP	Aerobic, semi dwarf, short duration, disease, coarse, non -scented
Sathi-Black	Farmers' Variety UP	Aerobic, semi dwarf, short duration, disease, coarse, non- scented
IR-74371-4-6-1-1	IRRI, the Philippines	Aerobic, semi dwarf, mid- duration, L S, non -scented
IR-71604-1-4-1	IRRI, the Philippines	Aerobic, semi dwarf, mid- duration, LS, non- scented
IR-71527-44-1-1	IRRI, the Philippines	Aerobic, semi dwarf, mid- duration, LS, non- scented
IR-74371-70-1-2	IRRI, the Philippines	Aerobic, semi dwarf, mid- duration, LS, non -scented
IR-74371-70-1-1	IRRI, the Philippines	Aerobic, semi dwarf, mid- duration, LS, non -scented
IR-80919	IRRI, the Philippines	Aerobic, semi dwarf, mid- duration, LS, non- scented
IR-75298-59-3-1-3	IRRI, the Philippines	Aerobic, semi dwarf, mid- duration, non -scented
IR-73888-1-2-7	IRRI, the Philippines	Aerobic, semi dwarf, mid- duration, non- scented
IR-75298-59-8-1-3	IRRI, the Philippines	Aerobic, semi dwarf, mid- duration, non- scented
IR-71700-247-1-1-2	IRRI, the Philippines	Aerobic, semi dwarf, mid- duration, LS, non- scented
BG-352	Chhattisgarh, India	Aerobic, semi dwarf, mid- duration, broader leaves, non- scented
BG-358	Chhattisgarh, India	Aerobic, semi dwarf, mid- duration, broader leaves, non- scented
BG-379-2	Chhattisgarh, India	Aerobic, semi dwarf, mid- duration, parrot green leaves, non-scented
DGI-380	Srilanka	Aerobic, semi dwarf, mid- duration, non- scented
DGI-155	Srilanka	Aerobic, semi dwarf, mid- duration, non -scented
DGI-152	Srilanka	Aerobic, semi dwarf, mid- duration, non -scented
DGI-296	Srilanka	Aerobic, semi dwarf, mid- duration, non- scented
DGI-154	Srilanka	Aerobic, semi dwarf, mid- duration, non -scented
BG-94-1	Chhattisgarh, India	Aerobic, semi dwarf, mid- duration, dark green, non -scented
BG-359	Chhattisgarh, India	Aerobic, semi dwarf, mid- duration, light green, non -scented

Irri: Adapted to Irrigated agro-eco system, Aerobic: Adapted to aerobic agro-eco system, LS: Long slender grain, Disease: Variety susceptible to diseases, TB: Traditional Basmati variety, EB: Evolved Basmati variety.

molecular characterization of rice germplasm (Wu and Tanksley, 1993; Yang et al., 1994). SSR markers are simple, PCR based, locus specific, more reliable, reproducible and typically co-dominant markers.

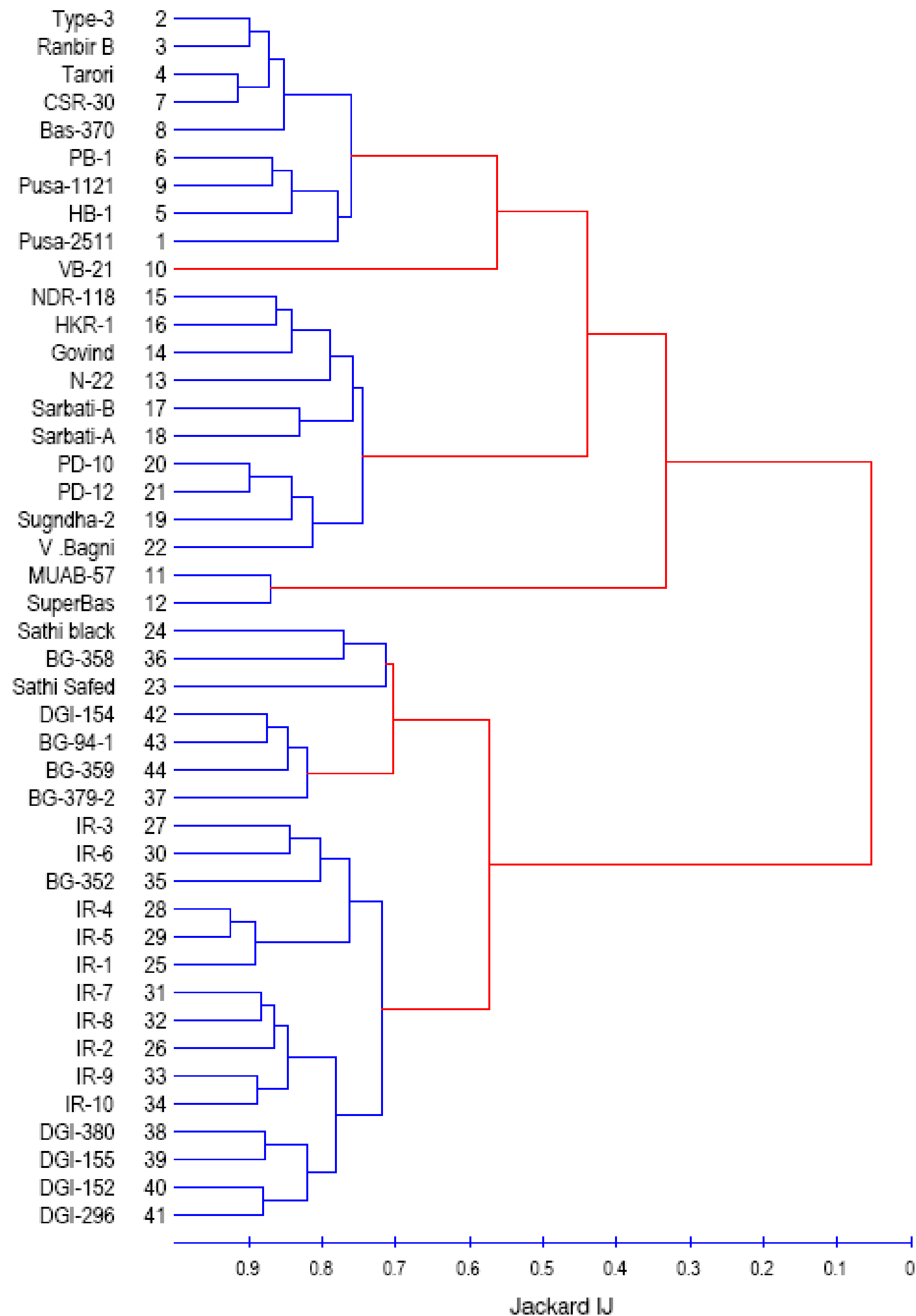

Figure 1. Dendrogram showing clustering of 44 rice varieties constructed using UPGMA based on Jacquard's similarity coefficient obtained from ISSR and SSR joint analysis. Ranbir B, Ranbir Basmati; Bas. 370, Basmati 370; PB-1, Pusa Basmati 1; HB-1, Haryana Basmati-1; VB-21, Vallabh Basmati-21; CSR-30, Basmati CSR-30; N-22, Nagina-22; PD-10, Pant Dhan-10; PD-12, Pant Dhan-12; V. Bagni, Vallabh Bangani; MAUB-57, Vallabh Basmati -23; Super Bas, Super Basmati.

The present data used for characterization of rice genotypes was generated involving ten ISSR, 20 eight SSR molecular markers and 44 Basmati and non-Basmati rice varieties. The 22 varieties of Basmati and non-Basmati rice adapted to Irrigated agro ecosystem considered presently were developed in Indo-Gangetic plains of Northern India (Singh et al., 2000a) where rice is cultivated mainly in Irrigated puddled conditions (puddle

that is, anaerobic conditions, the traditional method of rice cultivation in Northern India). The rest 22 rice genotypes collected from four different sources (1) IRRI, the Philippines (2) Sri Lanka (3) Central India (4) Indo-Gangetic plains of Northern India, were adapted to aerobic agro-ecosystem that is, direct seeding rice (unpuddled). Out of 22 varieties adapted to aerobic conditions, two varieties Sathi white and Sathi black were Farmers' varieties collected from Indo-Gangetic plains of Northern India. Both the Sathi strains/ farmers' varieties mature in 75 days (seed to seed) with minimal water requirement and remained in cultivation in aerobic conditions over decades in the area of their adaptation before deployment of irrigation facilities. Also, Vallabh Basmati-21, the variety recently released for Irrigated agro-eco system (Singh et al., 2011) has expressed excellent harvests in aerobic conditions (Singh, 2008).

In ISSR-PCR assay, all the 44 rice varieties were classified agro-ecosystem wise into two major distinct clusters. Twenty two (22) varieties of irrigated agro ecosystem were further grouped into 5 clusters based on variations in quality. Out of these, 11 varieties of super fine quality were distributed over 3 clusters depending upon minor variations in quality. Four traditional Basmati varieties Ranbir Basmati, Type 3, Basmati 370 and Taroari Basmati along with four evolved basmati varieties Pusa-1121, HB-1, CSR-30 and PB-1 were clustered together as also reported earlier by Nagaraju et al. (2002).

None of the variety was clustered with Vallabh Basmati 21 and therefore, it was put in a separate cluster. The improved dwarf genotype MAUB 57 recently identified as an elite genotype of Basmati rice for UP and Haryana by AICRP (Anonymous, 2007) and Super Basmati popular in Punjab were clustered together in a separate cluster consistently in all the three assays. The rest 11 varieties of non-basmati rice adapted to irrigated agro-eco system were grouped into two different clusters.

Furthermore, all the 22 varieties adapted to aerobic conditions were further grouped into two sub clusters. Three varieties BG 352, BG 358 and Sathi Black were grouped into one sub cluster. The rest 19 genotypes were distributed over five sub clusters. Within agro-eco systems, the distribution of varieties in the main as well as in sub clusters reflected independence of their geographical origin. The varieties of aerobic rice despite having their origin in the Philippines, Sri Lanka, Northern India (Sathi strains) and Central India were grouped in only one major cluster indicating presence of common base sequence repeats in the genomic DNA corresponding to ISSR markers used. Such DNA sequences could perhaps have evolved, due to different kinds of selection pressures required to acquire fitness well for survival, in due course of time. The explanation could also be forwarded that all the genotypes of common gene pool would have exchanged over the locations collected there from not long before. In other words, the time passed after shifting of such germplasm was too short to attain adaptation as a consequence of a new genetic mechanism which could have evolved in due course of time. Such sequences were detected by the molecular markers used herein. Therefore, it might be concluded that the base sequences which determined ISSR markers were corresponding to the gene complexes responsible for quality and adaptation reactions.

Examination of SSR-PCR assay reflected, minor variations in clustering pattern. However, clustering of the 8 varieties of basmati rice involving traditional and evolved groups remained the same. Likewise, both the evolved varieties of basmati rice MAUB-57 and Super Basmati were consistently grouped in a separate cluster. PS-2511 and Vallabh Basmati-21 adapted to irrigated agro ecosystem were clustered with varieties of aerobic rice, that is, Sathi Black, BG-375-2, and BG-358. Vallabh Basmati-21 being adapted to aerobic conditions might have genes common to those adapted to aerobic conditions.

However, it could not be detected by ISSR markers. It suggested that PS-2511 requires further evaluation in aerobic agro ecosystem for adaptation reactions. Additionally, it might be concluded that SSR markers were, in general, more robust and therefore, could detect even the minor variations more efficiently in the genomic DNA. Such variations are taken as a basis of molecular characterization of the genotypes (Wu and Tanksley, 1993; Yang et al., 1994).

Furthermore, the clustering pattern of joint assay was more similar to that of ISSR clustering pattern. However, it varied slightly when joint assay was taken into consideration for DNA profiling of these varieties (Figure 1). PS-2511, a super fine non Basmati variety was also included into the cluster containing traditional as well as evolved varieties of basmati rice. PS-2511 expressed quality characteristics of Basmati of inferior quality (Table 2) reflecting its limited closeness to Basmati group. This is why; despite continues efforts, PS- 2511 could not be popularized as Basmati among consumers. It also holds true in the present context as reflected by its inconsistent clustering with Basmati group. However, its molecular lineage with Basmati was detected only by ISSR+SSR joint assay (Figure 1). However, impact of adaptation and quality on classification of varieties remained distinct.

In parallel, all the genotypes suitable for aerobic conditions were clustered in a distinct major cluster almost consistently in all the three assays. More variation was noticed in clustering pattern, in general, among the varieties adapted to irrigated agro-eco system over all the three analyses. The varied minor genetic changes which have occurred spontaneously during course of evolution to acquire fitness in a particular agro-eco system seem to be the only cause. It could be an evolutionary adaptation that serves to discriminate between genotypes on the basis of their quality. Examination of dendrogram (Figure 1) and the results discussed above suggested that

Table 2. The component characteristics of rice quality (approved by Government of India and acceptable in the global trade) were scored at quality laboratories, DRR, Hyderabad (AP) and CRRI Cuttack (Orissa). For the sake of brevity, results only of some of basmati varieties Vallabh Basmati-21, MAUB-57, Taroari Basmati and Pusa Basmati-1 obtained at DRR, Hyderabad are given below.

Quality parameter	Quality standards approved by DAC, GOI and expressed by Basmati varieties						
	Approved by DAC, GOI and also acceptable in global trade	Quality parameters expressed by commercially popular and recently released/identified varieties of Basmati rice					
		PB-1	P S-2511	Haryana Basmati	Taroari Basmati	Vallabh Basmati-21	Vallabh Basmati-22
Milling %	65.0 (minimum)	67.0	71.0	70.4	69.2	66.2	69.37
Head rice recovery %	45.0 (minimum)	52.1	58.9	44.5	52.2	51.0	52.01
Kernel length (mm)	6.61 (minimum)	6.98	7.95	6.62	6.94	7.64	7.45
Kernel breadth (mm)	2.00 (maximum)	1.78	1.89	1.72	1.79	1.79	1.72
Length breadth ratio	3.5 (minimum)	3.88	4.21	3.85	3.88	4.27	4.30
Volume expansion ratio	3.0 (minimum)	4.85	3.75	5.50	4.63	4.7	4.74
Water uptake (ml)	250 (minimum)	362	315	285	268	310	205.50
Kernel length after cooking (KLAC) mm	12.0 (minimum)	15.5	14.4	11.0	13.3	13.3	14.80
Elongation ratio (ER)	1.70 (minimum)	2.22	1.8	1.51	1.92	1.73	1.98
Alkali Spreading Value (ASV)	4-7	7.0	7.0	7.0	5.0	7.0	5.08
Amylose content %	20-25	24.81	24.60	24.12	24.44	24.48	23.47
Gel Consistency (GC)	>61	466.6	47.0	47.0	49.66	47.00	54.66
Aroma score (1-3)	3.0	3.0	3.0	3.0	3.0	3.0	3.0

AICRP- All India Coordinated Research Project, DRR- Directorate of Rice Research, Hyderabad, India. CRRI- Central Rice Research Institute, Cuttack (Orissa), India. DAC- Department of Agriculture and Co-operation, GOI- Government of India.

clustering of 44 rice genotypes was mainly based on two factors that is, adaptation and quality (Table 1 and 2). Both adaptation and quality of rice are complex traits.

Adaptation is governed by several gene complexes. Adaptation is a function of the genes possessed by the variety for the regulation of biochemical and physiological processes during growth and development and how well these are matched with the available environmental resources and possible hazards. The adaptation results from a genetic mechanism which influences the matching of their growth and development processes within the environment (Fisher and Scott, 1993). Thus, process of adaptation is viewed as changes in the genetic constitution of genotypes as they accumulate genes or a change in gene frequencies within population which better match growth and development within the environment (Byth, 1981; Clements et al., 1983; Cooper and Hammer, 1996). Adaptation is more complicated but its elegant genetic studies are lacking. Quality of rice is the resultant effect of a number of component traits.

Contrarily, quality of Basmati rice is a complex character consisting of 13 physicochemical component characteristics (Table 2). In addition, finally quality is judged/ examined by a panel of experts. Each component character is governed by different genetic mechanisms. Comprehensive information about genetic mechanism of quality components is available in the literature and some of such studies are listed in the Table 3. Though, aroma is an essential component of Basmati. However, among non-Basmati rice genotypes such as PS- 2511 also carried aroma.

The study reveals that visibly distinguishable qualitative characters like plant height, photosensitivity, disease resistance, colour, size and number of awns, and sensory characters such as aroma etc. could not significantly influence clustering of genotypes. Therefore, the varieties differ for such characters could not be identified by using such molecular tools. For example, tall and dwarf collections of farmers' variety Sarbati could not be identified by the markers presently used and, therefore, were clustered together in a single cluster in all the three cases. Likewise, morphologically distinct genotypes MAUB-57 and Super Basmati were also clustered together. Such results indicated limited utility of molecular markers to the breeders and traders. It appears self-explanatory that small size of DNA sequences of genes responsible for such characters does not have significant bearing on the clustering of these varieties. DNA sequences of small size representing genes responsible for such characters could not be detected by the markers used. However, trait specific markers could be used to detect such characters. In such cases it seems easier to classify the varieties on the basis of morpho-physico-chemical characteristics and application of molecular markers seems of limited value. In the study, impact of any individual component character of quality on classification could not be noticed. None of the variety was classified on the basis of aroma alone. DNA

Table3. Studies describing genetic mechanisms of quality components of rice.

Characters	QTLs/Chrom. arm	References
Aroma	Aro3-1/3S, aro4-/4L, aro8-1/8L	Ahn et al.1992; Petrov et al., 1996; Lorieux et al., 1996; Wanchana et al., 2005; Chen et al., 2006; Amarawathi et al., 2008
Alkali spreading value	Asv6-1/6S	McKenzie and Rutger, 1983; He et al., 1999; Amarawathi et al., 2008
Amylose content	Amy6-1/6S	McKenzie and Rutger, 1983; He et al., 1999; Kumar and Khush, 1996; Amarawathi et al., 2008
Kernal elongation ratio	Elr11-1/11L	Ahn et al., 1993; Amarawathi et al., 2008
Length/breadth ratio	Lbr7-1/7L, Lbr7-2/7L	Redona and Mackill, 1998; Rabiei et al., 2004; Amarawathi et al., 2008
Grain breadth	Grd7-1/7L, gbr7-2/7L	Lin et al., 1995; Redona and Mackill, 1998; Sarkar et al., 1994; Amarawathi et al., 2008
Grain length	Grl11-1/1L, grl7-1/7Lgrl7-2/7L	Lin et al., 1995; Redona and Mackill, 1998; Aluko et al., 2004; Wan et al., 2006; Amarawathi et al., 2008
Gel consistency	qGC-4,qGC-6,qGC-4a,qGC-4b,qGC-5, qGC-8a, qGC-8b, qGC-12a, qGC-12b	Tan et al., 1999; He et al., 1999; Lanceras et al., 2000; Umemoto et al., 2002; Tian et al., 2005; Sabouri et al., 2009

Table 4. Primers sequence (SSR and ISSR).

Primer code		Sequence	Maker
RM 4	Forward	TTGACGAGGTCAGCACTGAC	IDT
	Reverse	AGGGTGTATCCGACTCATCG	
RM 5	Forward	TGCAACTTCTAGCTGCTCGA	IDT
	Reverse	GCATCCGATCTTGATGGG	
RM11	Forward	TCTCCTCTTCCCCCGATC	IDT
	Reverse	ATAGCGGGCGAGGCTTAG	
RM17	Forward	TGCCCTGTTATTTTCTTCTCTC	IDT
	Reverse	GGTGATCCTTTCCCATTTCA	
RM30	Forward	GGTTAGGCATCGTCACGG	IDT
	Reverse	TCACCTACCACACGACACG	
RM38	Forward	ACGAGCTCTCGATCAGCCTA	IDT
	Reverse	TCGGTCTCCATGTCCCAC	
RM42	Forward	ATCCTACCGCTGACCATGAG	IDT
	Reverse	TTTGCTCTAAGTGGCGTACA	
RM44	Forward	TCGGGAAAACCTACCCTACC	IDT
	Reverse	ACGGGCAATCCGCCCAACC	
RM51	Forward	TCTCGATTCAATGTCCTCGG	IDT
	Reverse	CTACGTCATCATCGTCTTCCC	
RM55	Forward	CCGTCGCCGTAGTAGAGAAG	IDT
	Reverse	TCCCGGTTATTTTAAGGCG	
RM84	Forward	TAAGGGTCCATCCACAAGATG	IDT
	Reverse	TTGCAAATGCAGCTAGAGTAC	
RM85	Forward	CCAAAGATGAAACCTGGATTG	IDT
	Reverse	GCACAAGGTGAGCAGTCC	
RM104	Forward	GGAAGAGGAGAGAAAGATGTGTGTCG	IDT
	Reverse	TCAACAGACACACCGCCACCGC	
RM107	Forward	AGATCGAAGCATCGCGCCCGAG	IDT
	Reverse	ACTGCGTCCTCTGGGTTCCCGG	
RM127	Forward	GTGGGATAGCTGCGTCGCGTCG	IDT
	Reverse	AGGCCAGGGTGTTGGCATGCTG	

Table 4. Contd.

RM128	Forward Reverse	AGCTTGGGTGATTTCTTGGAAGCG ACGACGAGGAGTCGCCGTGCAG	IDT
RM131	Forward Reverse	TCCTCCCTCCCTTCGCCCACTG CGATGTTCGCCATGGCTGCTCC	IDT
RM136	Forward Reverse	GAGAGCTCAGCTGCTGCCTCTAGC GAGGAGCGCCACGGTGTACGCC	IDT
RM141	Forward Reverse	CACCACCACCACCACGCCTCTC TCTTGGAGAGGAGGAGGCGCGG	IDT
RM154	Forward Reverse	ACCCTCTCCGCCTCGCCTCCTC CTCCTCCTCCTGCGACCGCTCC	IDT
RM160	Forward Reverse	AGCTAGCAGCTATAGCTTAGCTGGAGATCG TCTCATCGCCATGCGAGGCCTC	IDT
RM161	Forward Reverse	TGCAGATGAGAAGCGGCGCCTC TGTGTCATCAGACGGCGCTCCG	IDT
RM163	Forward Reverse	ATCCATGTGCGCCTTTATGAGGA CGCTACCTCCTTCACTTACTAGT	IDT
RM235	Forward Reverse	AGAAGCTAGGGCTAACGAAC TCACCTGGTCAGCCTCTTTC	IDT
RM252	Forward Reverse	TTCGCTGACGTGATAGGTTG ATGACTTGATCCCGAGAACG	IDT
RM273	Forward Reverse	GAAGCCGTCGTGAAGTTACC GTTTCCTACCTGATCGCGAC	IDT
RM302	Forward Reverse	TCATGTCATCTACCATCACAC ATGGAGAAGATGGAATACTTGC	IDT
RM330	Forward Reverse	CAATGAAGTGGATCTCGGAG CATCAATCAGCGAAGGTCC	IDT

Table 4. Contd.

Primer code	Sequence	Maker
ISSR-1F	5’GGCGGCGGCGGCGGCAT3’	IDT
ISSR-2F	5’AAGAAGAAGAAGAAGGC3’	IDT
ISSR-3F	5’AAGAAGAAGAAGAAGTG3’	IDT
ISSR-4F	5’AAGAAGAAGAAGAAGCC3’	IDT
ISSR-5C	5’AGCAGCAGCAGCAGCCA3’	IDT
ISSR-6F	5’AGCAGCAGCAGCAGCCG3’	IDT
ISSR-7F	5’GGCGGCGGCGGCGGCTA3’	IDT
ISSR-8F	5’AGCAGCAGCAGCAGCGA3’	IDT
ISSR-9F	5’AAGAAGAAGAAGAAGCG3’	IDT
ISSR-10F	5’CCAGTGGTGGTGGTG3’	IDT

sequences accounted for by QTLs responsible for several component characters of adaption as well as quality were spread over the whole genome and were detected by the corresponding molecular markers used. Therefore, respective genes responsible for some crucial components of quality and adaptation which greatly influenced clustering of varieties of both the agro-eco systems seem to be largely represented by ISSR and SSR markers’ DNA sequences. Clustering of the genotypes used herein greatly depended upon adaptation to IRRI gated and aerobic agro-eco systems within limits of regulations of the genetic mechanism evolved for adaptation, represented by ISSR and SSR sequences. Likewise, within irrigated agro-eco system quality determined classification of varieties.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Review

Oyster mushrooms (*Pleurotus*) are useful for utilizing lignocellulosic biomass

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This review shows the biotechnological potential of oyster mushrooms with lignocellulosic biomass. The bioprocessing of plant byproducts using *Pleurotus* species provides numerous value-added products, such as basidiocarps, animal feed, enzymes, and other useful materials. The biodegradation and bioconversion of agro wastes (lignin, cellulose and hemicellulose) could have vital implication in cleaning our environment. The bioprocessing of lignin depends on the potent lignocellulolytic enzymes such as phenol oxidases (laccase) or heme peroxidases (lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase) produced by the organism. The cellulose-hydrolysing enzymes (that is, cellulases) basically divided into endo-β-1,4-glucanase , exo-β-1,4-glucanase I and II, and β-glucosidase, they attack cellulose to release glucose, a monomers units from the cellobiose, while several enzymes acted on hemicellulose to give D-xylose from xylobiose. These enzymes have been produced by species of *Pleurotus* from lignocellulose and can also be used in several biotechnological applications, including detoxification, bioconversion, and bioremediation of resistant pollutants.

Key words: Oyster mushroom, lignin, cellulose, hemicellulose.

INTRODUCTION

Lignocellulosic materials are the most promising feedstock as natural and renewable resource essential to the functioning of modern industrial societies (Anwer et al., 2014). A large amount of such materials as waste by-products are being generated through agricultural practices mainly from various agro based industries (Pe´rez et al., 2002).

Large amounts of lignocellulosic waste generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agro-industries, and they are posing serious environmental pollution problems (Howard et al., 2003). Accidentally, much of the lignocellulosic biomass is often disposed of by burning or just lying values without any important used attached to them.

Recently, lignocellulosic biomasses have gained increasing research interests and special importance because of their renewable nature (Asgher et al., 2013; Ofori-Boateng and Lee, 2013). This has attracted the interest of many researchers in the utilization of lignocel-

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lulosic wastes. *Pleurotus* are characterized by a white spore print, attached to the gills, often with an essentric stip, or no stip at all, and they are commonly known as Oyster mushrooms (Miles and Chang, 1997). Growing oyster mushroom is becoming more popular throughout the world because of their abilities to grow at a wide range of climate conditions and utilizing various lignocelluloses. In nature, *Pleurotus* species live on parts of plants which are generally poor in nutrients and vitamins.

For spawn running and fruit body development, lignin and cellulose materials such as corn cobs, all cereal straws, paper, wood shavings, sawdust, nutshells and vegetable wastes as well as food industry wastes are sufficient (Baysal et al., 2003). Species of the genus *Pleurotus* (Fr.) P. Kumm known as oyster mushrooms, rank third place in worldwide production of edible mushrooms after *Agaricus bisporus* and *Lentinula edodes*, and comprises various edible species with medical, biotechnological and environmental applications (Cohen et al., 2002). *Pleurotus* species present high adaptability to produce basidiomata within a wide variety of agro-industrial lignocellulosic wastes due to their production of ligninolytic and hydrolytic enzymes (Mikiashvili et al., 2006).

Agro-residues contain three major structural polymers, cellulose, hemicellulose and lignin, which can be easily utilized or broken down by the lignocellulotic enzymes. *Pleurotus* species are the most efficient lignin-degrading organisms, with the ability to produce mainly laccases (EC 1.10.3.2), lignin peroxidase (EC 1.11. 10.14) and manganese peroxidase (EC1.11.1.13) (Adebayo et al., 2012a). These enzymes present a non-specific biocatalyst mechanism and have been used for bioremediation process due to their ability to degrade azo, heterocyclic, reactive and polymeric dyes (Baldrian and Snajdr, 2006; Forgacs et al., 2004). White-rot basidiomycetes are among the most potent organisms to biodegrade and detoxify a wide range of wastes and pollutants. These fungi selectively attack lignin and related compounds by producing one or more of phenol-targeting redox enzymes, namely the peroxidases and laccases/phenol-oxidases (Ntougias et al., 2012). Prospection for fungi is the ability to secret high levels of lignin-degrading enzymes and novel enzyme variants, with desirable properties for biotechnological applications (Adebayo et al., 2012a). Therefore, the huge amounts of lignocellulosic biomass can be potentially bioconverted into different high value raw materials and products such as bio-ethanol, enriched animal feed, cheap energy sources for microbial cultivation (mushrooms included) and enzyme production, biodegradation and bioremediation of toxic organic compounds (Koutrotsios et al., 2014; Anwar et al., 2014; Asgher et al., 2013; Irshad et al., 2013; Ntougias et al., 2012). The objectives of this review are the compilation of the newer achievements in the technologies developed between oyster mushrooms and lignocellulosic materials.

CHEMICAL COMPOSITIONS OF LIGNOCELLULOSE

Lignocellulosic biomass are the most abundant and less utilized biomass, they includes agricultural wastes, forestry residues, grasses and woody materials, which are good source of substrates for oyster mushroom and in turn resulted into valuable products. Agricultural lignocellulosic biomass basically consists of 40 to 50% cellulose, 25 to 30% hemicellulose and 15 to 20% lignin (Malherbe and Cloete, 2002; Iqbal, et al., 2011; Menon and Rao, 2012). Major structural component of plant cell walls is cellulose, which is responsible for its mechanical strength. Hemicelluloses are macromolecules with repeated polymers of pentoses and hexoses, while lignin contains three aromatic alcohols (coniferyl alcohol, sinapyl alcohol and pcoumaryl alcohol) produced through a biosynthetic process and forms a protective seal around the other two components i.e., cellulose and hemicelluloses (Figure 1) (Sanchez, 2009; Menon and Rao, 2012; Anwar et al., 2014). Composition of lignocellulose is generally depends on its source; whether it is derived from the hardwood, softwood, or grasses. Table 1 shows the typical chemical compositions in cellulose, hemicellulose and lignin from various lignocellulosic materials. The variation in chemical compositions may be due to the genetic variability among different sources (Iqbal et al., 2011; Menon and Rao, 2012: Kumar et al., 2009; Malherbe and Cloete, 2002). The chemical formula of cellulose is $(C_6H_{10}O_5)_{n,}$ with structure of single chain polymer shown in Figure 1.

Cellulose is a highly stable polymer consisting of glucose and attached with linear chains up to 12,000 residues. It is majorly com-posed of (1,4)-D-glucopyranose units, which are attached by β-1,4 linkages with an average molecular weight of around 100,000 (Himmel et al., 2007). Cellulose held together by intermolecular hydro-gen bonds in native state, but they have a strong tendency to form intra-molecular and intermolecular hydrogen bonds and this tendency increases the rigidity of cellulose and makes it highly insoluble and highly resistant to most organic solvents. Naturally, cellulose molecules exist as bundles which aggregated together in the form of micro-fibrils order that is, crystalline and amorphous regions (Iqbal et al., 2011; Taherzadeh and Karimi, 2008).

Hemicellulose mainly consists of glucuronoxylan, glucomannan and trace amounts of other polysaccharides. They are majorly found in grasses and straws contain arabinan, galactan and xylan, while mannan is a component of hardwood and softwood (Brigham et al., 1996). Hemicelluloses have sugar backbone which composed xylans, mannans and glucans, with xylans and mannans being the most common (Wyman et al., 2005). Galactans, arabinans and arabinogalactans are also

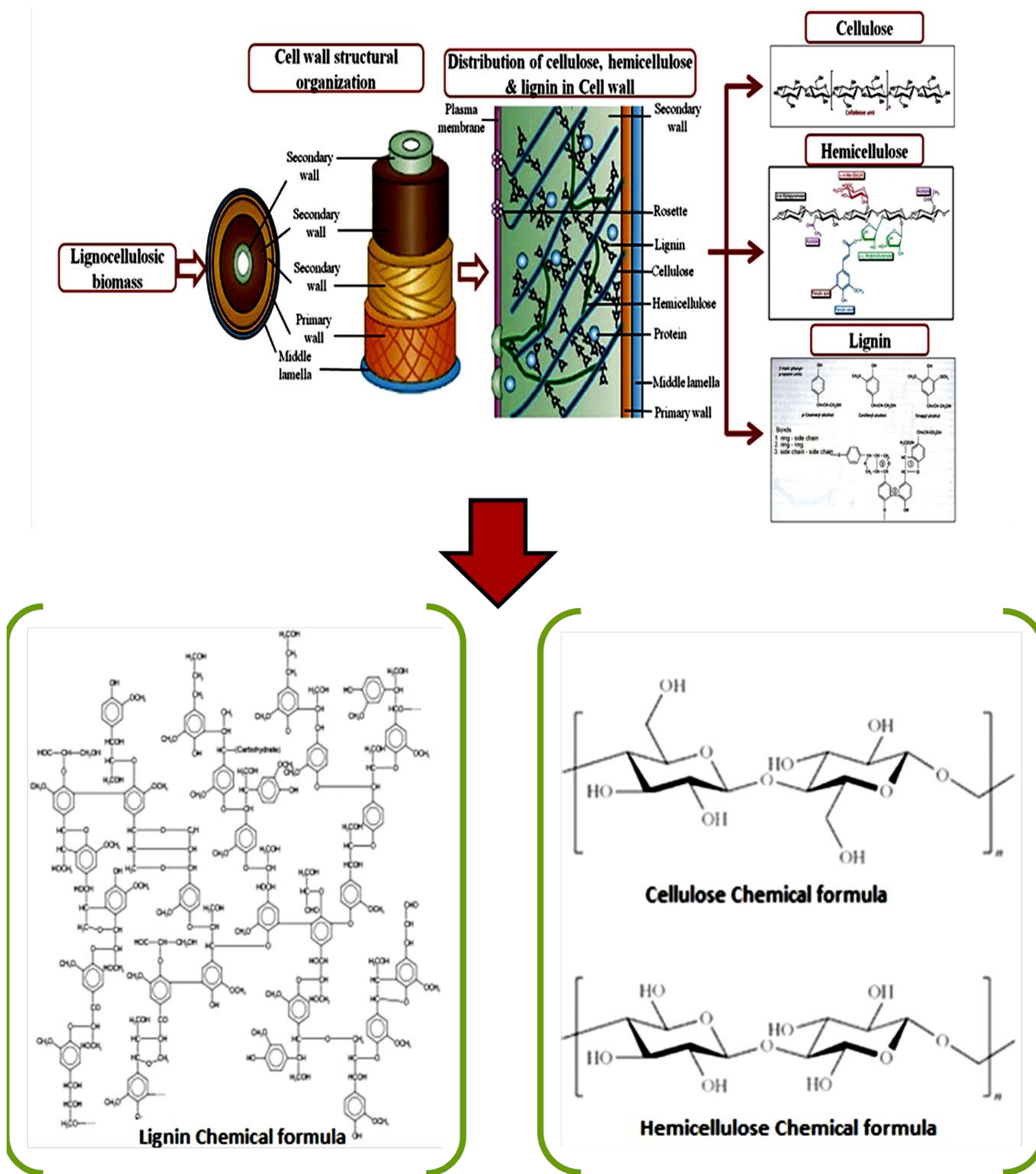

Figure 1. Diagrammatic illustration of the framework of lignocellulose; lignin, cellulose and hemicellulose.

included in the hemicellulose group; however, they do not share the equatorial β-1,4 linked backbone structure. In hardwoods, glucuronoxylan (O-acetyl-4-O-methyl-glucurono-b-Dxylan) is the predominant component, xylospyranose is the backbone of the polymer and connected with β-1,4 linkages. Hemicellulose has average molecular weight of <30,000. Cellulose and hemicellulose binds tightly with non-covalent attractions

Table 1. Composition of representative lignocellulosic feed stocks.

Feedstock	Carbohydrate composition (% dry wt)			References[a]
	Cellulose	Hemicellulose	Lignin	
Barley hull	34	36	19	Kim et al., 2008
Barley straw	36-43	24-33	6.3-9.8	Garda-Aparricio et al., 2006
Bamboo	49-50	18-20	23	Alves et al., 2010
Banana waste	13	15	14	Monsalve et al., 2006
Corn cob	32.3-45.6	39.8	6.7-13.9	Mckendry, 2002
Corn stover	35.1-39.5	20.7-24.6	11.0-19.1	Mosier et al., 2005
Cotton	85-95	5-15	0	Kadolph and Langford, 1998
Cotton stalk	31	11	30	Rubio et al., 1998
Coffee pulp	33.7-36.9	44.2-47.5	15.6-19.1	Perez-Diaz et al., 2005
Douglas fir	35-48	20-22	15-21	Schell et al., 1999
Eucalyptus	45-51	11-18	29	Alves et al., 2010
Hardwood stems	40-55	24-40	18-25	Howard et al., 2003
Rice straw	29.2-34.7	23-25.9	17-19	Prassad et al., 2007
Rice husk	28.7-35.6	11.96-29.3	15.4-20	Abbas and Ansumali, 2010
Wheat straw	35-39	22-30	12-16	Abbas and Ansumali, 2010
Wheat bran	10.5-14.8	35.5-39.2	8.3-12.5	Miron et al., 2001
Grasses	25-40	25-50	10-30	Hon, 2000
Newspaper	40-55	24-39	18-30	Howard et al., 2003
Sugarcane bagasse	25-45	28-32	15-25	Singh et al., 2009
Olive tree biomass	25.2	15.8	19.1	Cara et al., 2008
Sugarcane tops	35	32	14	Jeon et al., 2010
Pine	42-49	13-25	3-29	Pereira, 1998
Poplar wood	45-51	25-28	10-21	Torget and Hsu, 1994
Jutefibres	45-53	18-21	21-26	Mosihuzzaman et al., 1982
Switchgrass	35-40	25-30	15-20	Howard et al., 2003
Grasses	25-40	25-50	10-30	Malherbe and Cloete, 2002
Winter rye	29-30	22-26	16.1	Petersson et al., 2007
Oilseed rape	27.3	20.5	14.2	Petersson et al., 2007
Softwood stem	45-50	24-40	18-25	Howard et al., 2003
Oat straw	31-35	20-26	10-15	Rowell, 1992
Nut shells	25-30	22-28	30-40	Sinner et al., 1979
Sorghum straw	32-35	24-27	15-21	Vazquez et al., 2007
Tamarind kernel powder	10-15	55-65	ND	Menon et al., 2010
Water hyacinth	18.2-22.1	48.7-50.1	3.5-5.4	Aswathy et al., 2010

ND = Not determined, [a]For detailed references: Menon and Rao (2012). Source: Menon and Rao (2012).

to the surface of each cellulose micro-fibril. Hemicelluloses were originally believed to be intermediates in the biosynthesis of cellulose (Vercoe et al., 2005).

Lignin has a long-chain, heterogeneous polymer composed largely of phenyl-propane units, most commonly linked by ether bonds. Lignin acts like a glue by filling the gap between and around the cellulose and hemicellulose complexion with the polymers. It is present in all plant biomass and comprised of complex and large polymer of phenyl-propane, methoxy groups and noncarbohydrate poly phenolic substance, which bind cell walls component together (Hamelinck et al., 2005).

OYSTER MUSHROOMS

Oyster mushrooms are commercially important in the world mushroom market, and several species are grown commercially on a large and small scale in many countries (Adebayo et al., 2012c). *Pleurotus* species have been recognized as mushroom with dual functions to humans; both as food and medicine (Chang and Buswell, 2003). They are nutritive with good quantity of proteins, vitamins and minerals. Medicinally, they are been recommended for obese persons and diabetes patients because of low calorie value (Chang and Buswell, 2003) and very low sugar without starch.

Traditionally, extracts from *Pleurotus* species have been reported to be used in treating some ailments (Osemwegie et al., 2010; Idu et al., 2007). *Pleurotus* are preeminent wood decomposers; they grow on a wider array of forest and agricultural wastes than species of any other group. They thrive on most all hardwoods, on wood by-products (sawdust, paper, pulp sludge), all the cereal straws, corn and corn cobs, on sugar cane bagasse, coffee residues (coffee grounds, hulls, stalks and leaves), banana fronds, cottonseed hulls, agave waste, soy pulp and on other materials too numerous to mention and difficult to imagine possible. In cultivating oyster mushrooms, several valuable by-products are generated. After the crop cycle is complete, the remaining substrate is rendered into a form animal feeds such as cattle, chickens and pigs. Using the spent straw as a nutritious food source could help replace the wasteful practice of feeding grain in the dairy and cattle industry (Stamets, 2000).

Taxonomy of genus *Pleurotus*

Oyster mushrooms are cosmopolitan, and belong to the genus *Pleurotus* (Fungi: Basidiomycetes). Their cap is normally shell-like (about 5 to 20 cm in diameter; 1.9 to 7.8 inches), fleshy, with eccentric or lateral stipe; and their color can be white, cream, yellow, pink, brownish, or dark gray (Martínez-Carrera, 1999). Oyster mushroom was first cultivated by 1917 in Germany by Flank. Evolutionary connection among species in the genus *Pleurotus* is still not clear and many taxonomic issues remain controversial. The genus *Pleurotus* is one of the most diverse groups of cultivated mushrooms. Fungal populations are established and developed through sexual and asexual reproduction (Cohen et al., 2002). Conventional methods for classification (fruit body morphology, microscopic observations, mating studies between populations, biochemical analyses) have not provided clear-cut results (Martínez-Carrera, 1999). This taxonomic confusion has always been associated with the genus *Pleurotus*, especially species belonging to the *Pleurotus ostreatus* complex due to morphological variations of different specimens and similarity of isolates belonging to different species (Asef, 2012). Molecular studies have shown to be more informative; intra and interspecific heterogeneity was determined using ribosomal and mitochondrial DNA analyses, and phylogenetic studies of ribosomal DNA sequences indicated geographic speciation in several groups. Hilber (1982) reported results of mating reactions as well as the microscopic and macroscopic characteristics of many *Pleurotus* isolates and concluded that *Pleurotus pulmonarius* and *P. ostreatus* were different species, *Pleurotus sapidus* is more likely to correspond to *Pleurotus cornucopiae,* and *Pleurotus columbinus* is simply a variety of *P. ostreatus*. There are other disagreements concerning the exact taxonomic position of the cultivar *Pleurotus florida* (considered either as a *P. pulmonarius* or a *P. ostreatus* strain) and whether *Pleurotus cystidiosus* and *Pleurotus abalonus* belong to separate (Han et al., 1974; Bresinsky et al., 1987) or the same species (Jong and Peng, 1975; Moore, 1985). Zervakis and Labarer (1992) classified 23 isolates of genus *Pleurotus* with dendrogram of taxonomic distances demonstrated the suitability of enzyme isoelectric focusing, as it clearly distinguished the four well-defined species *P. cornucopiae, P. cystidiosus, Pleurotus eryngii and Pleurotus flabellatus.* The *P. ostreatus*, whose taxonomy is controversial, was separated from *P. pulmonarius*, and *P. columbinus* was classified as a distinct taxon. The delimitation of *Pleurotus sajor-caju* and *P. sapidus* strains appeared to be more difficult as they seemed to be closely related, the former with *P. pulmonarius* and the latter with *P. ostreatus*. Vilgalys et al. (1993) identified three intersterile groups, *P. ostreatus, P. pulmonarius* and a new species limited to North America: *Pleurotus populinus.* Vilgalys et al. (1996) identified 15 intersterility groups of *Pleurotus* (*ostreatus, pulmonarius, populinus, cornucopiae, djamor, eryngii, cystidiosus, levis, dryinus, tuberregium, agaves, abieticola, brazil, australis, purpureo-olivaceus*) of which some groups have been added in the later time. Oyster mushroom are generally classified as follow; Scientific Name: *Pleurotus* spp.; Phylum: *Basidiomycotina*; Class: *Basidiomycetes*; Subclass: *Holobasidiomycetidae*; Family: *Polyporaceae*; Genus: *Pleurotus;* Species: *sajor caju*, *sapidus, ostreatus, eous, membranaceous, florida, citrinopileatus, flabellatus, pulmonarius* etc.

Life cycle of *Pleurotus* (Oyster mushroom)

Pleurotus mushrooms show the typical life cycle of Basidiomycetes, a major fungal group (Figure 2). It begins with the germination of a basidiospore in a suitable substrate, which gives rise to a monokaryotic mycelium containing genetically identical nuclei (n) and capable of indefinite independent growth. When two compatible monokaryotic mycelia are in close contact, they are able to establish a fertile dikaryon by hyphal fusion or plasmogamy. This dikaryon (n+n), having clamp connexions and binucleate in each hyphal compartment, contains two genetically different nuclei (one from each monokaryon) throughout the mycelium. When environmental conditions are appropriate (temperature, light, relative humidity), the dikaryotic mycelium will differentiate into fruit bodies having specialized structures called basidia. In these club-shaped, binucleate cells, which are formed in the lamellae (hymenium) of each fruit body, karyogamy (fusion of the paired nuclei; 2n) and meiosis (recombination and segregation) take place. The four resulting haploid nuclei move to the sterigmata on the basidium, to form four new basidiospores. When the

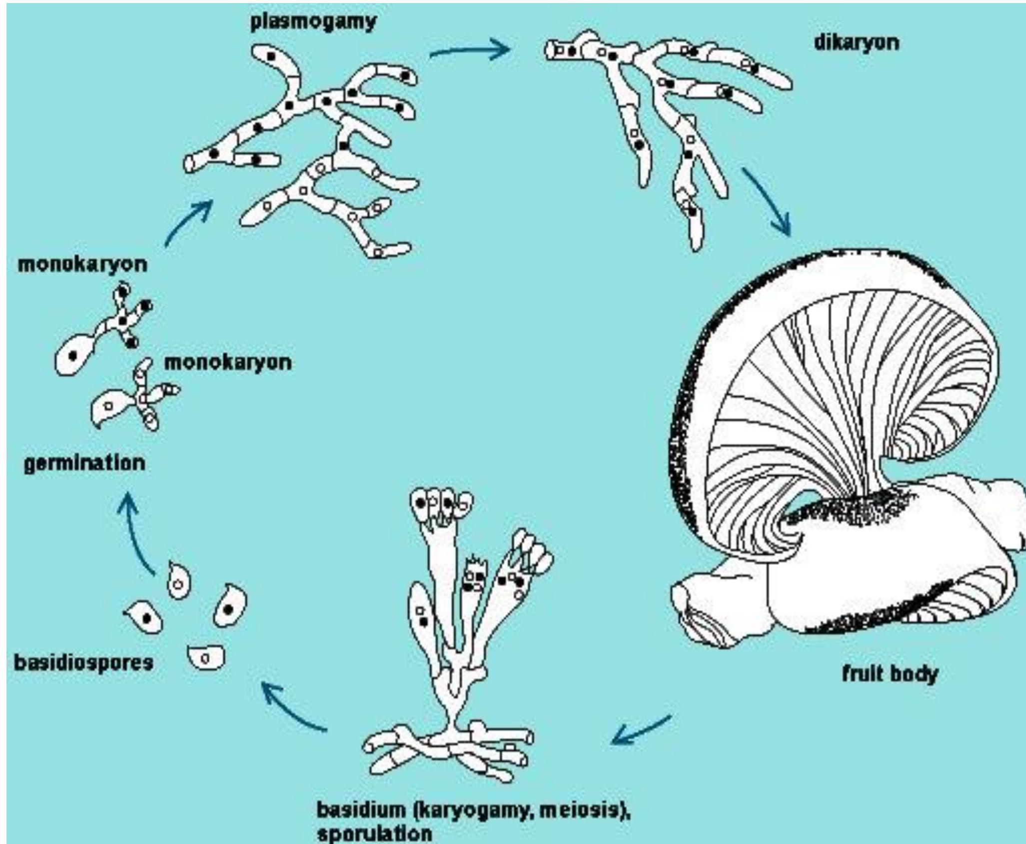

Figure 2. Life cycle of the oyster mushroom *Pleurotus ostreatus*. Source: Martínez-Carrera, 1999.

fruit bodies are mature, basidiospores are discharged, starting the sexual life cycle again.

Lignocellulose biomass as substrate for *Pleurotus* cultivation

In commercial cultivation of mushroom, the availability of good substrates is major limiting factor, which determine mushrooms growth rate and quality of yield produced. The cost of mushroom is directly dependent on the substrate availability and utilizable potential of mushroom species. *Pleurotus* species have been known to grow on a wider array of forest and agricultural wastes than any other mushroom species. Cultivation of edible mushroom with agricultural residues is a value- added process to convert these materials, which otherwise considered to be a wastes, into human and animal food (Zhang et al., 2002). It represents one of most efficient biological ways by which these residues can be recycled (Madan et al., 1987). Oyster mushrooms (*Pleurotus* spp.) are in the third place after the white button and shiitake among the world mushroom production (Gyorfi and Hajdu, 2007). Due to a large variety of non-specific lignocellulosic enzymes produced by them, they can be cultivated on a number of agricultural wastes (Zhang et al., 2002). Varieties of agrowastes have been used in cultivating oyster mushroom, with most of them proved promising and enhanced the yield performance (Table 2). Amongst various cereal straws, paddy straw was reported to be the best substrate for the cultivation of oyster mushroom (Bano and Srivastava, 1962; Jandaik and Kapoor, 1974; Khanna and Garcha, 1982), whereas, next to the paddy straw, wheat straw proved to be the best substrate for the cultivation of *Pleurotus* species (Bano and Rajarathnam, 1982; Bhatti et al., 1987; Thampi et al., 1996; Bonatti et al., 2004). Sorghum straw was also effectively used to cultivate *P. sajor-caju* (Bahukhandi and Munjal, 1989; Patil et al., 1989). Similarly, Garcha et al. (1984) and Diwakar et al. (1989) reported the utility of pearl millet stalks in the cultivation of *P. sajor-caju*. Rye straw waste (Pal and Thapa, 1979), lawn grass (Yamashita et al., 1983), maize cobs (Bhatti et al., 1987), banana waste (Bonatti et al., 2004) and maize straw (Bahukhandi and Munjal, 1989) were reported as suitable substrates for cultivations of different *Pleurotus* species. Bhandari et al. (1991) successfully cultivated *P. sajor-caju* on straws of millets. Many other types of substrates were also reported to be useful for the cultivation of various species of *Pleurotus* species as shown in Table 2.

Table 2. Yield performance of *Pleurotus* spp. on various agro-residues

Substrate	PP (day)	Total yield (g)	BE (%)	References
Paddy straw	29	1,093	109.30	Adebayo et al., 2013
Wheat straw	22	341	68.2	Siddhant et al., 2013
Saw dust	48	205	86.85	Poppe, 1995
Banana leaves	32	770	134.60	Bhavani and Nair, 1989
Guniea grass	36	405	121.04	Kiran and Jandaik, 1989
Coconut leaves	31	585	107.25	Theradi, 1992
Sorghum stalks	29	885	122.45	Patil et al., 2008
Sugarcane bagasse	30	545	125.80	Khan and Ali, 1982
Newspaper	20	1010	193.65	Poppe, 1995
Maize stalks	26	725	139.20	Ruhul Amin et al., 2007
Maize stover	29	ND	89.97	Ragunathan et al., 1996
Coir Pith	27	ND	94.42	Ragunathan et al., 1996
Guar straw	ND	108	11.66	Ragini et al., 1987
Bajra straw	ND	100	7.21	Ragini et al., 1987
Jowar straw	ND	108	7.66	Ragini et al., 1987
Bagasse	ND	97	8.33	Ragini et al., 1987
Sarkanda leaves	ND	73	7.15	Ragini et al., 1987
Mango leaves	ND	61	5.96	Ragini et al., 1987
Coffee pulp	ND	999	159.9	Martínez-Carrera, 2000
Palm oil husk	ND	190	63.3	Adebayo et al., 2014
Cotton Waste	ND	ND	56.41	Martínez-Carrera, 2000

ND = Not determined.

BIOCONVERSION OF AGRO- WASTE INTO USEFUL PRODUCTS

Conversion of lignocellulose into food and feed rich in protein by fungi offers an alternative for developing unconventional source of proteins as food/feed (Mane et al., 2007). Large quantity of lignocellulose materials are produced as by-products and its accumulation in the environment could result to environmental problem. The utilization of waste (reuse or recycle) is very important, in order to keep our environment free of any obnoxious condition. Alternative methods of the utilizing these agricultural wastes are needed to mitigate the environment pollution problems associated with current disposal methods, such as open-field burning and soil incorporation. *Pleurotus* species as primary wood rot fungi are able to colonize different types of agricultural wastes as substrates. Thus, they are cultivated on different lignocellulose wastes. Exploitation of the substrate varies with the species, strain and cultivation technology (Zadrazil and Dube, 1992). In fact, the prospect of utilization of such largely unexploited materials is significant, especially with the cultivation of mushroom to generate new value-added products. Rumen microorganisms convert cellulose and other plant carbohydrates in large amounts to acetic, propionic and butyric acids, which ruminant animals can use as energy and carbon sources (Ezeji et al., 2006; Martin et al., 2006; Albores et al., 2006); these microbes also have potential for commercial bioprocessing of lignocellulosic wastes anaerobically in liquid digesters.

Studies on combination of an integrated system of composting, with bio-inoculants (strains of *P. sajor-caju, Trichoderma harzianum, Aspergillus niger* and *Azotobacter chroococcum*) and subsequent vermicomposting showed an accelerated composting process of wheat straw, besides producing a nutrient-enriched compost (Singh and Sharma, 2002). Koutrotsios et al. (2014) reported a high reduction of both fibre and the content of hemicelluloses and cellulose of spent cultivation substrate of *P. ostreatus*, an increase in crude protein of spent substrate is also reported compared to initial substrate (Table 3).

ENZYMES PRODUCED BY *PLEUROTUS* SPECIES IN SEVERAL LIGNOCELLULOSE BIOMASS

Basidiomycetes fungi especially *Pleurotus* species are the most efficient lignin-degrading organisms that produce mainly laccases (EC 1.10.3.2), lignin peroxidase (EC 1.11.10.14) and manganese peroxidase (EC1.11.1.13) (Adebayo et al., 2012a). These enzymes present a non-specific biocatalyst mechanism and have been used for bioremediation process due to their ability to degrade azo, heterocyclic, reactive and polymeric dyes

Table 3. Selected constituents of nine (spent) cultivation substrates by *Pleurotus ostreatus*.

Parameter	AN	BS	CC	GM	OL	OS	PL	PN	WS
N	0.5	0.0	0.5	2.7	1.8	1.1	1.2	2.3	0.5
K	72	ND	424	1057	758	1164	1328	307	364
Na	ND	2.1	12.8	43.6	27.0	21.4	52.6	8.5	14.2
P	46.8	39.5	62.3	193.3	90.9	74.1	121.8	288.8	66.9
Ash	1.7	1.8	3.7	12.5	14.7	4.1	15.0	28.0	13.0
Crude protein	3.1	0.3	3.0	17.1	11.6	6.6	7.5	14.2	2.8
Crude fat	3.7	0.3	0.3	3.2	2.1	0.8	0.2	2.02	0.5
Total carbohydrate	91.6	97.7	93.3	75.2	74.6	78.9	77.7	68.4	68.6
Crude fibre	41.0	50.9	36.7	23.2	25.2	49.5	31.5	11.6	32.9
Hemicellulose	18.4	22.1	20.7	4.1	8.8	15.8	14.3	1.1	12.9
Lignin	16.6	11.0	6.1	39.8	18.3	21.0	14.7	23.8	7.4
Cellulose	28.8	48.7	46.1	11.3	12.7	20.4	26.9	3.9	39.3
Residual biomass	148.3	159	157.4	86.9	103.3	93.1	140	114.9	136.9

ND= Not determined; Source: Koutrotsios et al. (2014). AN, almond and walnut shells 1:1 w/w; BS, beech sawdust; CC, corn cobs; GM, grape marc plus cotton gin trash 1:1 w/w; OL, olive mill by-products (leaves and two phase olive mill waste 1:1 w/w); OS, extracted olive-press cake; PN, pine needles; PL, date palm tree leaves and WS: wheat straw after the production. Values (% d.w., except of the first three parameters) are expressed as means.

(Baldrian and Snajdr, 2006; Forgacs et al., 2004). Fungi prospection is the ability to secret high levels of lignin-degrading enzymes and novel enzyme variants, with desirable properties for biotechnological applications. On the other hand, alternative low cost substrates like agricultural residues for enzyme production using solid state fermentation (SSF) offer economic and environmental advantages. Different types of agro-industrial wastes have been reported from literature as raw materials for diverse value-added products. The cost of raw materials in enzymes production, which translates into 40 to 60% has make enzymes production generally expensive (Hacking, 1987). In this perspective, the utilization of wastes as growth substrates for the production of enzymes is more economical and profitable. Agro-industrial wastes were omnipresence, high biodegradability, and rich in carbon with optimum potential to serve as substrate for enzymes production. Several species of *Pleurotus* have been isolated and identified as producer of lignocellulotic enzymes with better performance on agro waste as substrate (Table 4).

Biodegradation of lignocellulosic biomass

Lignocellulose degrading mushroom species are used in various SSF applications such as biodegradation of hazardous compounds and biological detoxification of toxic agro-industrial wastes (Soccol and Vandenberghe, 2003; Philippoussis and Diamantopoulou, 2011), bio-transformation of agro-industrial residues to mushroom food and animal feed (Philippoussis, 2009), compost and product developments such as biologically active metabolites, enzymes, food, flavour compounds and other added value compounds (Philippoussis and Diamantopoulou, 2011). It has been reported that some species of *Pleurotus* are able to colonize different types of vegetable wastes, increasing their digestibility (Villas-Boas et al., 2002; Zhang et al., 2002; Mukherjee and Nandi, 2004).

Salmones et al. (2005) reported that three species of *Pleurotus* consumed lignocellulose with varied degradation percentage from each strain. In particular *P. ostreatus* efficiently degraded hemicellulose on coffee pulp and wheat straw and lignin on wheat straw, whereas *Pleurotus djamor* showed a preference for the cellulose of coffee pulp. *P. pulmonarius* was distinguished by a high consumption of lignin when grown on coffee pulp. Generally, greater decrease in lignocellulosic compounds was observed for wheat straw samples than for coffee pulp (Salmones et al., 2005). The cellulose has been reported as the most biodegraded component of the lignocellulose by fungi due to its content of the substrate in the production of fruiting bodies (Geetha and Sivaprakasam, 1998; Thomas et al., 1998), followed by the hemicellulose which is less abundant than the cellulose and least is the lignin (Salmones et al., 2005).

Generally, *Pleurotus* species follow the mechanism employed by white-rot fungi in degrading lignocellulose waste. White-rot fungi degrade lignin by secreting enzymes collectively termed “ligninases”. Ligninases can be classified as either phenol oxidases (laccase) or heme peroxidases [lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP) (Martinez et al., 2005). Production of lignin peroxidases (EC1.11.1.14) (LiP), manganese peroxidases (EC 1.11.1.13) (MnP) and laccases (EC 1.10.3.2) from *P. ostreatus* and *P. pulmonarius* have been studied (Okamoto

Table 4. Enzymes produced by *Pleurotus* species in several agricultural wastes.

***Pleurotus* species**	**Substrates**	**Enzyme**	**References**
P. ostreatus	Bagasse of cane maize straw	Xylanases, cellulase, Laccase, MnP, LiP.	Marquez at al. (2007) Okamoto et al. (2002)
P. ostreatus, P. pulmonarius	Coffee pulp, used nappy, grass residues, wheat straw, Industrial cotton fiber.	Endoglucanase, cellobiohydrolase, Laccase, MnP, LiP.	Marnyye et al. (2002) Okamoto et al. (2002)
P. ostreatus, P. citrinopileatus	Wheat straw	Laccase, MnP, Endo- β -1,4-glucanase Endo- β -1,4-xylanase β-Glucosidase, β -xylosidase	Carabajal et al. (2012)
P. tuber-regium	Cotton waste, rice straw, cocoyam peels, corncob, groundnut shell, sawdusts of *Khaya ivorensis*, *Mansonia altissima* and *Boscia angustifolia* coffee husks, eucalyptus sawdust, eucalyptus bark, with or without 20 % rice bran	Cellulase, Proteinase, amylase, α-Amylase, Carboxymethylcellulase, Lipase, Peroxidase, Catalase, Polyphenol oxidase, Glucose-6-phosphatase.	Kuforiji and Fasidi (2008)
P. ostreatus		MnP, laccase, LiP, cellulases, xylanases, tanases.	Luz et al. (2012).
P. eryngii	Liquid substrate	Versatile peroxidase Lip, MnP	Camarero et al. (1999)
P. ostreatus, P. eryngii	Liquid substrate	versatile peroxidase Aryl-alcohol oxidase	Ruiz-Duenas et al. (1999) Cohen et al. (2002)
P. sajor-caju, P. florida	Used tea leaves	Aryl-alcohol dehydrogenases Laccase, Lip.	Guillen et al. (1992)
P. ostreatus, P. sajor-caju	saw dust, paddy straw, sugarcane bagasse	Cellobiohydrolase β-glucosidase, CMCase.	Rana and Rana (2011)
P. florida, P. sajor- sajor	Sugarcane leaves, paddy and Wheat straw	Endo-β-1; 4-glucanase, Exo-β-1; 4-glucanase, β-glucosidase, cellulases	Khalil et al. (2011) Ortega et al. (1993)

et al., 2002; Carabajal et al., 2012; Luz et al., 2012), while VPs have been reported to be produced by *P. eryngii* (Camarero et al., 1999; Ruiz-Duenas et al., 1999) and *P. ostreatus* (Cohen et al., 2002) (Table 4).

Lignin biodegradation

Laccase

Laccases are glycosylated blue multi-copper oxidoreductases (BMCO) that use molecular oxygen to oxidize various aromatic and nonaromatic compounds through a radical catalyzed reaction mechanism (Claus, 2004; Baldrian, 2006). Laccases couple the electron reduction of dioxygen into two molecules of water with the oxidation of a vast variety of substrates, such as phenols, arylamines, anilines, thiols and lignins (Figure 3a) (Thurston, 1994). Four copper ions in their catalytic center mediate the redox process. These are classified as being type-1 (T1), type-2 (T2) and two type-3 (T3 and T3’), based on the copper’s coordination and spectroscopic properties (Messerschmidt and Huber, 1990). The oxidation reactions catalyzed by laccases lead to the formation of free radicals which act as intermediate substrates for the enzymes (Figure 3b) (Ferraroni et al., 2007). These mediators can leave the enzyme site and react with a broad range of high-redox potential substrates and thus create non-enzymatic routes of oxidative polymerizing or depolymerizing reactions (Figure 1c) (Dashtban et al., 2010). Ultimately, laccase-mediator system (LMS) becomes involved in a range of physiological functions such as lignolysis (Figure 3d), lignin synthesis, morphogenesis, pathogenesis and

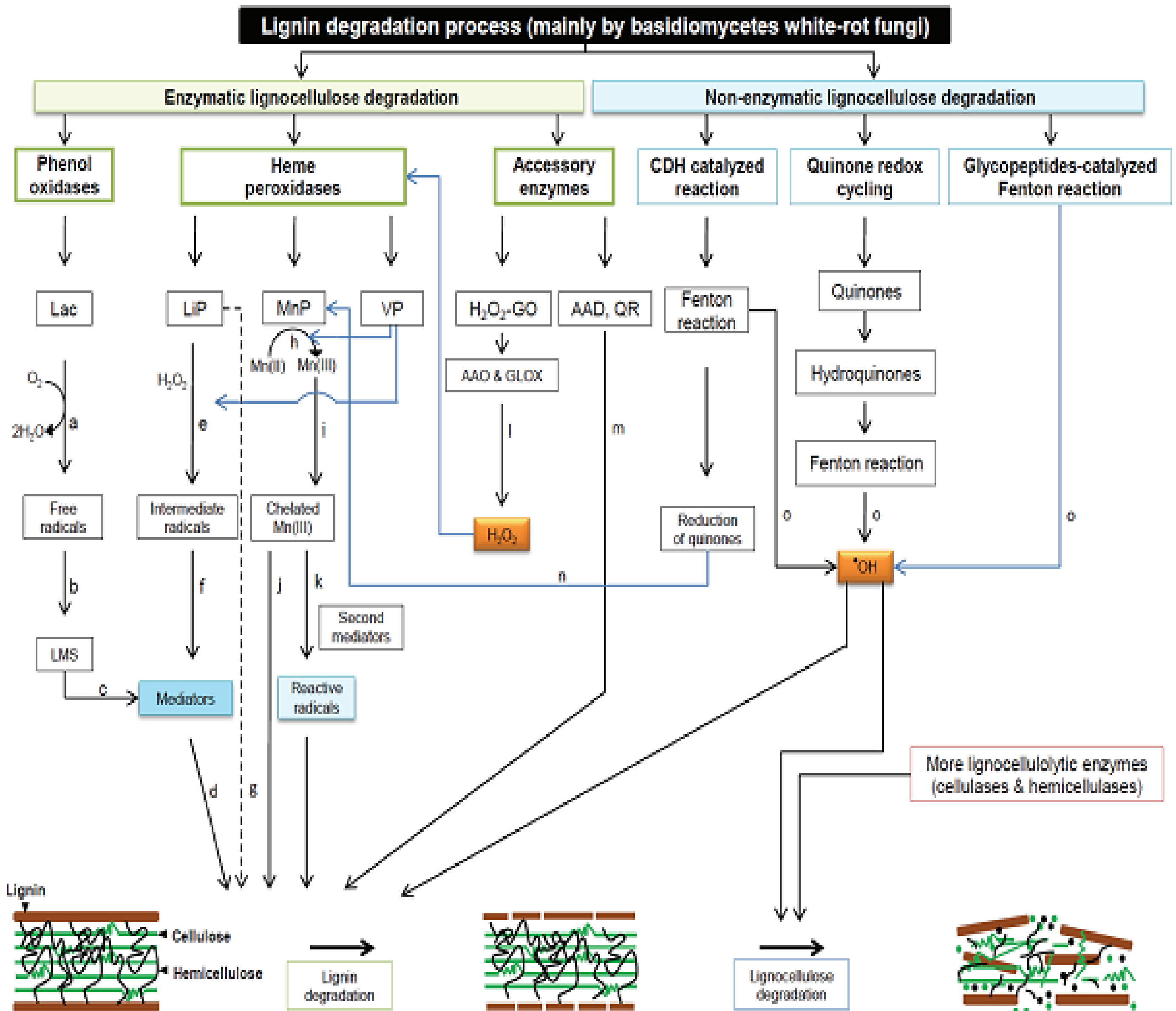

Figure 3. Schematic diagram of lignin degradation by basidiomycetes white-rot fungi: the major steps and enzymes involved (refer to text). Lac: laccase-mediator system, LiP: lignin peroxidase, MnP: manganese peroxidase, VP: versatile peroxidase, H_2O_2-GO: H_2O_2-generating oxidase, AAO: aryl-alcohol oxidase, GLOX: glyoxal oxidase, H_2O_2: hydrogen peroxide, AAD: aryl-alcohol dehydrogenases, QR: quinone reductases and OH: free hydroxyl radicals. Source: Dashtban et al. (2010).

detoxification (Mayer and Staples, 2002).

Heme peroxidase

Lignin peroxidases (LiP): Lignin peroxidase is one of the heme-containing glycoproteins and plays a major role in the biodegradation of lignin cell wall constituent (Piontek, 2001). LiPs catalyze the H_2O_2-dependent oxidative depolymerization of a variety of non-phenolic lignin compounds (diarylpropane), β-O-4 non-phenolic lignin model compounds and a wide range of phenolic compounds (e.g. guaiacol, vanillyl alcohol, catechol, syringic acid, acteosyringone) (Wong, 2009). LiPs oxidize the substrates in multi-step electron transfers and form intermediate radicals, such as phenoxy radicals and veratryl alcohol radical cations (Figure 3e). These intermediate radicals undergo non-enzymatic reactions such as radical coupling and polymerization, side-chain cleavage, demethylation and intramolecular addition and rearrangement (Figure 3f) (Wong, 2009). Unlike the other peroxidises, LiP is able to oxidize non-phenolic aromatic

substrates and does not require the participation of mediators due to its unusually high redox potential (Figure 3g) (Wong, 2009; Wang et al., 2008). The crystal structure of the first LiP has shown that the heme group is buried in the interior of the protein and has access to the outer medium through a channel (Dashtban et al., 2010). Although, the size of the channel is not sufficient to allow the large polymer lignin to access the heme group, small molecule substrates can find a suitable binding site (Piontek, 2001).

Manganese peroxidases (MnP): Manganese Peroxidases are extracellular glycoproteins and are secreted in multiple isoforms which contain one molecule of heme as iron protoporphyrin IX (Asgher et al., 2008). MnP catalyzes the peroxide dependent oxidation of Mn (II) to Mn (III) (Figure 3h), which is then released from the enzyme surface in complex with oxalate or with other chelators (Figure 3i). Chelated Mn (III) complex acts as a reactive low molecular weight, diffusible redox-mediator (Figure 3, j) of phenolic substrates including simple phenols, amines, dyes, phenolic lignin substructures and dimers (Wong, 2009; Wesenberg et al., 2003; Asgher et al., 2008). The oxidation potential of Mn (III) chelator is only limited to phenolic lignin structures. However, for the oxidation of non-phenolic substrates by Mn (III), reactive radicals must be formed in the presence of a second mediator (Figure 3k). Organic acids, such as oxalate and malonate are the primary compounds that act as second mediators in the production of reactive radicals like carbon centered radicals (acetic acid radicals, $COOHC{\bullet}H_2$), peroxyl radicals ($COOH\text{-}CH_2OO{\bullet}$), superoxide ($O_2{\bullet}\text{-}$) and formate radicals ($CO_2{\bullet}\text{-}$) (Wong, 2009; Wesenberg et al., 2003; Asgher et al., 2008). In the absence of H_2O_2 (e.g. in fungi lacking H_2O_2-generating oxidases), these radicals can be used by MnP as a source of peroxides and increase the lignin-degrading efficiency of the fungi (Wong, 2009; Wesenberg et al., 2003; Asgher et al., 2008).

Versatile peroxidases (VP): Versatile Peroxidases are glycoproteins with combine properties capable of oxidizing typical substrates of other basidiomycetes peroxidases including Mn (II) and also veratryl alcohol (VA), MnP and the typical LiP substrate, respectively (Figure 3) (Wesenberg et al., 2003; Asgher et al., 2008; Ruiz-Duenas et al., 1999). VPs form an attractive ligninolytic enzyme group due to their dual oxidative ability to oxidize Mn (II) and also phenolic and non-phenolic aromatic compounds (Wesenberg et al., 2003). It has been found that VPs can also efficiently oxidize high redox-potential compounds such as dye Reactive Black 5 (RB5) as well as a wide variety of phenols, including hydroquinones (Gomez-Toribio et al., 2001). It has been suggested that VPs can oxidize substrates spanning a wide range of potentials, including low- and high-redox potentials. This is a result of their hybrid molecular structures which provide multiple binding sites for the substrates (Camarero et al., 1999). This makes VPs superior to both LiPs and MnPs, which are not able to efficiently oxidize phenolic compounds in the absence of veratryl alcohol or oxidize phenols in the absence of Mn (II), respectively (Ruiz-Duenas et al., 1999). Similar to the MnP mechanism, Mn (III) is released from VPs and acts as a diffusible oxidizer of phenolic lignin and free phenol substrates (Figure 3h, i and j). Like other members of heme peroxidases, heme is buried in the interior of the protein and has access to the outer medium through two channels (Camarero et al., 1999). The function of the first channel is similar to that described for LiP and is conserved among all heme peroxidases. Conversely, the second channel is found to be specific to VP and MnP and is where the oxidation of Mn (II) to Mn (III) takes place (Ruiz-Duenas et al.,1999).

Other enzymes and mechanisms involved in lignin degradation

In addition to ligninases, other fungal extracellular enzymes which act as accessory enzymes have been found to be involved in lignin degradation. These include oxidases generating H_2O_2, which provide the hydrogen peroxide required by peroxidases, and mycelium-associated dehydrogenases, which reduce lignin-derived compounds (Figure 3l) (Martinez et al., 2005). Oxidases generating H_2O_2 include aryl-alcohol oxidase (AAO) (EC 1.1.3.7) found in *P. eryngii* (Guillen et al., 1992). In addition, aryl-alcohol dehydrogenases (AAD) (a flavoprotein) and quinone reductases (QR) are also involved in lignin degradation by *P. eryngii* (Figure 3, m) (Guillen et al., 1992). Moreover, it has been shown that cellobiose dehydrogenase (CDH), which is produced by many different fungi under cellulolytic conditions, is also involved in lignin degradation in the presence of H_2O_2 and chelated Fe ions (Henriksson et al., 2000). It is proposed that the effect of CDH on lignin degradation is through the reduction of quinones, which can be used by ligninolytic enzymes or the support of a Mn-peroxidase reaction (Figure 3n) (Henriksson et al., 2000). Previous studies have shown the involvement of non-enzymatic mechanisms in plant cell-wall polysaccharide degradation. Mechanisms are mostly assisted by oxidation through the production of free hydroxyl radicals (•OH). Many white and brown-rot fungi have been shown to produce hydrogen peroxide (H_2O_2) which enters the Fenton reaction and results in release of •OH (Figure 3o) (Guillen et al., 1992; Suzuki et al., 2006). By attacking polysaccharides and lignin in plant cell walls in a non-specific manner, these radicals create a number of cleavages which facilitate the penetration of the cell wall by lignocellulolytic enzymes (Call and Mücke, 1997; Wang

et al., 2006). Pathways by which fungi generate free •OH radicals are: cellobiose dehydrogenase (CDH) catalyzed reactions, low molecular weight peptides/quinone redox cycling and glycopeptide-catalyzed Fenton reactions (Renganathan et al., 1990).

Cellulose biodegradation

Cellulose is a homopolysaccharide composed of β-D-glucopyranose units, linked by β-(1→4)-glycosidic bonds. Cellulose contains both nonreducing (NR) and reducing (R) ends. The smallest repetitive unit of cellulose is cellobiose, which can be converted into glucose residues. The cellulose-hydrolysing enzymes (that is, cellulases) have been reported to be produced by species of *Pleurotus* from lignocellulose (Kuforiji and Fasidi, 2008; Carabajal et al., 2012; Luz et al., 2012) (Table 4). The enzyme (cellulases) divided into three major groups: endo-β-1,4-glucanase, exo-β-1,4-glucanase I and II, and β-glucosidase. The endo-β-1,4-glucanase catalyse random cleavage of internal bonds of the cellulose chain, while exo-β-1,4-glucanase I attack the chain at reducing ends to release cellobiose, and exo-β-1,4-glucanase II attack cellulose at nonreducing end chain. β-glucosidases are only active on cellobiose, and release glucose monomers units from the cellobiose (Figure 4A).

Hemicellulose biodegradation

The second most abundant renewable biomass which accounts for 25 to 35% of lignocellulosic biomass is hemicellulose (Saha, 2000). They are heterogeneous polymers built up by pentoses (D-xylose, L-arabinose), hexoses (D-glucose, D-galactose), sugar acids (Ferulic acid and 4-*O*-methyl-D-glucuronic acid and acetyl group. Many enzymes are responsible for the degradation of hemicellulose. The α-D-glucuronidase attack 4-*O*-methyl-D-glucuronic acid, while endo-β-1,4- xylanase break the xylan chains and α-L- arabinofuranosidase attack end chain of L-arabinose. The further reactions are cutting off ferulic acid and removing of the acetyl groups by feruloyl esterase and acetylxylan esterase, respectively. Reduction of xylan to xylobiose is done by α-D-galactosidase. The β-D-xylosidase release D-xylose, a monomers unit from xylobiose (Figure 4B). Like cellulose, hemicellulose is also an important source of fermentable sugars for biorefining applications (Figure 5). Xylanases are being produced and used as additives in feed for poultry and as additives to wheat flour for improving the quality of baked products at the industrial scale.

CONCLUSION

The challenges facing the whole world about the increased production of lignocellulloses materials from agricultural and forestry practices without efficient utilisation, has informed the alternative uses for this natural and renewable resources through a biotechnological manipulation of oyster mushroom. Majority of developing countries are still grappling with socio economic issues including food security, developing technological solutions in the agriculture, agro-processing and other related issues. Lignocellulolytic fungi, especially *Pleurotus* species, have attracted a great deal of interest as potential biomass degraders for large-scale applications due to their ability to produce vast amounts of valuable products and extracellular lignocellulolytic enzymes. Agro-waste materials which are majorly composed of lignin, cellulose and hemicellulose serves as major source of carbon and energy for *Pleurotus* species cultivation. Oyster mushroom and their enzymes serve as an efficient alternative for the biodegradation and bioconversion of lignocelluloses and other resistant pollutants. Lignocellulose biotechnology by oyster mushroom could produce numerous value-added products such as fruit body, extracellular enzymes, animal feed and other products. The bioprocessing of lignin depends on the potent lignocellulolytic enzymes such as phenol oxidases (laccase) or heme peroxidases (lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase) produced by the organism. The cellulose-hydrolysing enzymes (that is, cellulases) basically divided into endo-β-1,4-glucanase, exo-β-1,4-glucanase I and II, and β-glucosidase, they attack cellulose to release glucose, a monomers units from the cellobiose, while several enzymes (α-D-glucuronidase, , endo-β-1,4- xylanase, α-L- arabinofuranosidase, feruloyl esterase, acetylxylan esterase, α-D-galactosidase and β-D-xylosidase) acted on hemicellulose to give D-xylose from xylobiose. The bioconversion of lignocelluloses majorly involves transformation of lignin, hydrolysis of cellulose and hemicellulose to simple sugar, which can then play vital role in fermentation process. This bioprocess sometime refers to as fractionation.

In fact, the prospect of utilisation of such largely unexploited materials is significant, especially when accomplished by combining environmentally sound management with the generation of new value-added products. An indicative case complying with such prerequisites is the controlled solid state fermentation of various plant residues leading to the production of edible mushrooms. The productions of the protein rich foods at low cost could solve the problem of insufficient supply of quantity and quality of the bodybuilding material (protein) in feeding man.

Beside, that the enzymes produced serves in biodegradation or bioconversion of agro-waste, they can also be used in several biotechnological applications, including detoxification, bioconversion and bioremedia-tion of resistant pollutants. Several of these enzymes have previously reported of having the ability to degrade and

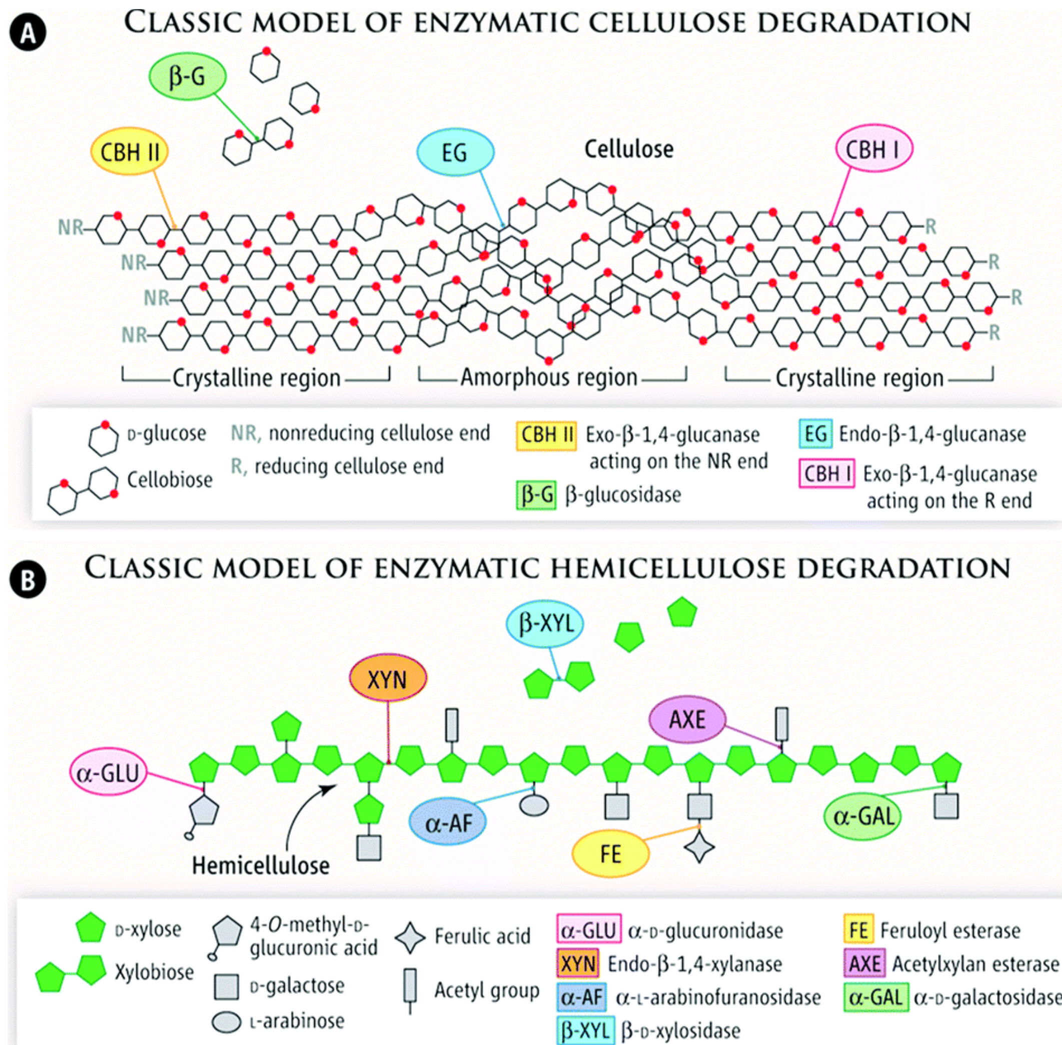

Figure 4. Model of enzymatic degradation with site of action in cellulose and hemicellulose.

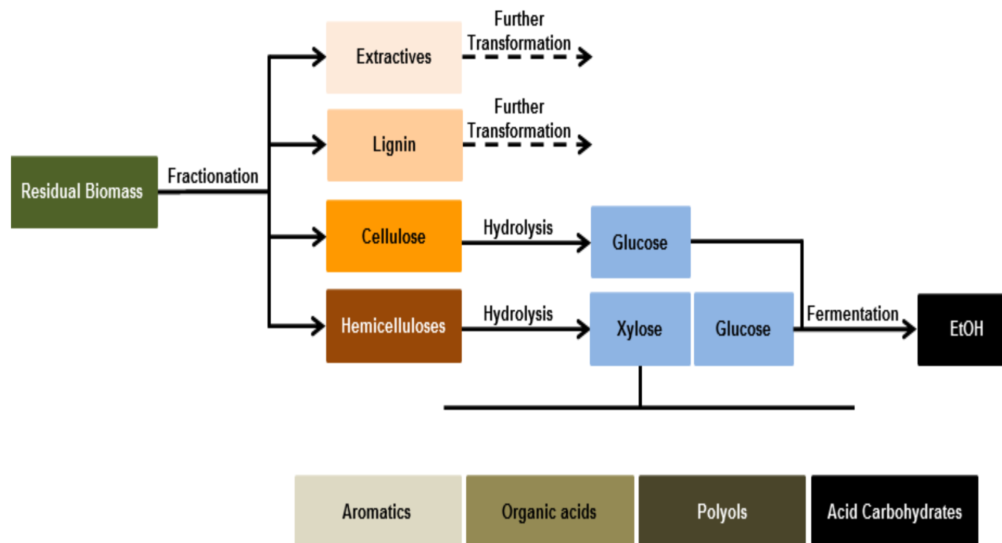

Figure 5. Different chemical reaction in bioconversion of lignocellolosic biomass.

mineralize toxic chemicals, such as polycyclic aromatic hydrocarbons (PAHs), atrazine, organophosphorus and wastewaters.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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