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Isolation and Screening of Potential Lignocellulolytic Microorganisms from Rubber Bark and Other Agricultural Residues

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Abstract

Lignocellulolytic microorganisms were isolated from cow manure, the soil from naturally grown bamboo, the piles of rubber bark and the composting piles. The samples were collected at 9 different locations which were 5 cm deep from the surface. The basal medium (BM) containing CMC (carboxymethyl cellulose) or xylan as a carbon source and the lignin modifying basal medium (LBM) with ABTS {2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid} or DMP (2,6-dimethoxyphenol) as an inducer were used for the primary screening of microorganisms producing cellulase, xylanase, laccase and manganese peroxidase, respectively. Forty-five bacterial isolates (B), twenty-five isolates of actinomyces (A) and fifteen fungal isolates (F) were obtained. After that twelve isolates were selected to evaluate enzyme production in LBM containing decomposed rubber bark as a carbon source at 30 °C and at 45 °C under aerobic conditions (150 rpm shaking). The isolates F6, A2 and B15 displayed the highest enzyme activities with a CMCase activity of 0.61, 0.33 and 0.21 U/mL, respectively. Moreover, they also produced xylanase with an activity of 0.61, 0.36 and 0.32 U/mL, respectively. However, only the isolate F6 showed laccase activity and manganese peroxidase activity of 0.81 and 0.56 U/mL, respectively. The isolates B15 and A2 were identified by 16S rDNA with 99 % similarity to *Bacillus subtilis* and *Streptomyces thermovulgaris*, respectively. Aside from that, isolate F6 was identified by 18S rDNA with 100 % similarity to *Trichoderma asperellum*. When these microorganisms were grown in the LBM broth with 10 % decomposed rubber bark they produced the highest lignocellulolytic enzymes in the stationary phase of growth.

Keywords: Cellulase, compost, ligninase, lignocellulolytic microorganisms, rubber bark

Introduction

Biomass residue from rubber trees is one potential biomass in Thailand. After the economic lifetime of rubber trees for latex production, which is around 25 - 30 years, they are cut down before replantation [1]. The rubber wood is used mainly as a raw material for furniture and wood based panel industries. After processing, there is a significant amount of rubber wood residues (sawdust, chips, bark) [2]. The major component of rubber wood residues is lignocellulose which consists of 30.66 % cellulose, 13.37 % hemicelluloses and 24.90 % lignin [3].

The making of compost from rubber bark and other agro-industrial and agricultural residues is an important alternative method for sustainable waste management in Thailand. Normally, the compost production is caused by mesophilic and thermophilic microbial activities [4]. They are involved in the composting process by excreting several extracellular enzymes including cellulase, xylanase and ligninase, etc. These enzymes are synthesized during the degradation of biomass. However, it takes a long time to degrade the lignocellulolytic wastes by natural processes. Therefore, inoculation of suitable

microbial strains into an initial biomass would speed up the composting process [5]. Many researchers have tried to find the microbes that have the ability to degrade lignocellulolytic wastes. *Anoxybacillus* sp. MGA110 is a thermophilic cellulolytic bacterium isolated from municipal solid waste compost. When it was inoculated into a compost pile it could degrade organic matter quicker than the control [6]. Rashad *et al*. [7] reported the enhancement of decomposition of rice straw compost inoculated with *Trichoderma reesei*, NRRL 11236 and *Phanerochaete chrysosporium*, NRRL 6361. Sharma *et al*. [8] screened 49 isolates of cellulolytic and xylanolytic organisms from rural compost. Among all the isolates, 4 bacterial isolates showed high cellulase and xylanase production and were identified as *Bacillus* sp., *B. licheniformis*, *B. megaterium* and *B. subtilis.* Wang *et al*. [9] isolated 50 thermophilic bacteria and found that 2 cellulose-effectively-decomposing thermophilic bacteria were identified as *Geobacillus* sp. and *Aeromanas* sp. from 13 composting piles and one soil sample.

The objective of this study was to isolate and screen the lignocellulolytic enzyme producing microorganisms from rubber bark and other agricultural residues. Afterwards, promising isolates were used to accelerate the composting process of rubber bark.

Materials and methods

Materials used

Fresh and decomposed rubber bark were obtained from the Rattaphum Sawmill Limited Partnership, Rattaphum, Songkhla, Thailand. All other chemicals used were of analytical grade.

Media for isolation and screening of lignocellulolytic microorganisms

Nutrient agar (NA) consisted of (g/l) beef extract, 3.0; peptone, 5.0; agar, 15.0 and cycloheximide, 0.2 (g/L) which was added to inhibit fungal growth. Actinomycete isolation agar (AIA) was composed of (g/L) sodium caseinate, 2.0; asparagine, 0.1; sodium propionate, 4.0; K_2HPO_4, 0.5; $MgSO_4$, 0.1; $FeSO_4$, 0.001; agar, 15.0; glycerol, 5.0 and cycloheximide, 0.2. Potato dextrose agar (PDA) consisted of (g/L) potato extract, 4.0; dextrose, 20.0; agar, 15.0 and chloramphenicol, 0.05 (g/L) which was added to inhibit bacterial growth. Lignin modifying basal medium (LBM) broth consisted of (g/L) K_2HPO_4, 1.6; KH_2PO_4, 0.5; $MgSO_4{\cdot}7H_2O$, 0.58; NaCl, 0.25; $CaCl_2{\cdot}2H_2O$, 0.013; $(NH_4)_2SO_4$, 1.25; NH_4NO_3, 1.0 and $FeCl_3{\cdot}6H_2O$, 0.0025 [10]. Basal medium (BM) contained the following constituents (g/L): K_2HPO_4, 0.5; $C_4H_{12}N_2O_6$, 0.5; $MgSO_4{\cdot}7H_2O$, 0.5; $CaCl_2{\cdot}2H_2O$, 0.01; $CuSO_4{\cdot}5H_2O$, 0.001; $Fe(SO_4)_3$, 0.001; $MnSO_4$, 0.001; yeast extract, 0.001 and agar 15.0 [11]. The cellulolysis basal medium (CBM) consisted of 2 % carboxymethyl cellulose (CMC) in BM and the xylanolysis basal medium (XBM) consisted of 2 % oat spelt xylan in BM [11]. ABTS agar and DMP agar were LBM agar with 0.1 % ABTS {2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)} and 0.01 % DMP (2,6-dimethoxyphenol), respectively. These 2 media were used for primary screening of laccase and lignin peroxidase producing microorganisms [11].

Characteristics of rubber bark and other agricultural and agro-industrial residues

Preparation of rubber bark

Both fresh and decomposed rubber bark were ground by the grinder to an average size of 0.5 mm and dried at 105 °C for 24 h, then stored in a plastic bag at 30 °C. The cellulose, hemicellulose and lignin compositions of rubber bark was analyzed for acid detergent fiber (ADF), neutral detergent fiber (NDF) and acid detergent lignin (ADL) according to Omar *et al*. [12].

The total nitrogen (TN) of the rubber bark was determined using the manual for organic composts analysis [13]. Total phosphorus (TP) and total potassium (TK) were analyzed using $HNO_3/HClO_4$ digestion according to the manual for organic composts analysis [13]. The moisture content of the rubber bark was determined based on weight loss after drying at 105 °C for 24 h [13]. The pH and electrical conductivity (EC) were measured in an aqueous suspension of a 1:10 ratio of rubber bark to water using a pH meter and a conductivity meter, respectively. Ash content was determined by burning the sample at 600 °C for 3 h [13]. The organic carbon (OC) and organic matter (OM) were analyzed using the Walkley-Black method [14].

The compositions of other agricultural and agro-industrial residues including lutoid, activated sludge, shrimp head, rice bran, bat guano and grease were also analyzed in the same way as rubber bark.

Isolation of bacteria, actinomyces and fungi

The samples for isolation of lignocellulolytic microorganisms were collected from different sources of agricultural and agro-industrial residues including the composting piles which consisted of decomposed rubber bark, lutoid, activated sludge and grease from the Green Tech 074 Company Limited, Hat Yai, Songkhla, Thailand; the piles of rubber bark from the Sri Trang Agro-Industry Pubic Company Limited, Hat Yai, Songkhla, Thailand; the cow manure from Na Mom, Songkhla, Thailand and the soil from naturally grown bamboo from Na Thawi, Songkhla, Thailand. The samples were collected at 9 different locations 5 cm deep from the surface. Fifty grams of sample were added to 450 mL of sterile 0.85 % NaCl. The suspension was mixed and allowed to settle 10 s. One milliliter of the liquid solution was serially diluted to 10^{-6}. Then 0.1 mL of this liquid mixture was spread evenly on an agar plate of different culture media, NA for bacteria, AIA for actinomyces and PDA for fungi. The plates were incubated at 30 °C and at 45 °C for mesophilic and thermophilic microbes, respectively. The various colonies were selected based on their morphology, size and color appearance on agar plates. These isolated organisms were purified by repeated streaking on the same medium. The purified isolates were kept on agar slant at 4 °C for further study.

Primary screening of lignocellulolytic microorganisms

The isolates were screened for lignocellulose degradation potential by the agar plate assay method.

Screening of cellulolytic and xylanolytic microorganisms

Pure cultures of microbial isolates were individually spotted on CBM and XBM agar plates. After 2 days of incubation at 30 °C and 45 °C, the plates were flooded with Gram's Iodine solution (2.0 g KI and 1.0 g iodine in 300 mL distilled water) for 3 to 5 min. Clear zones that appeared around microbial colonies indicated cellulose hydrolysis and xylan hydrolysis, respectively [15].

Screening of ligninolytic microorganisms

The microbial cultures were separately spotted on ABTS and DMP agar plates and incubated at 30 °C and 45 °C for 2 days. Production of laccase was determined by the formation of a green color on the ABTS agar. The formation of a yellow color on DMP agar was the positive reaction of lignin peroxidase [16]. The intense zone on the plate medium was measured and calculated as a potency index by using the following formula [17];

Potency index (PI) = diameter of clear zone or intense zone (mm) / diameter of colony (mm) (1)

The appearance of the zone around the colony indicated the production of the lignocellulolytic enzyme. The colony showing the largest PI was selected to determine its enzyme activity.

Secondary screening of lignocellulolytic microorganisms

Four isolates of bacteria, actinomyces and fungi that showed high PI were inoculated in 100 mL of LBM broth supplemented with 10 % of the decomposed rubber bark as a carbon source in a 250 mL Erlenmeyer flask. Each flask was inoculated with 10 mL of an inoculum (10^8 CFU/mL of bacteria or actinomyces and 10^8 spores/mL of molds). The cultures were incubated at 30 °C and at 45 °C with shaking at 150 rpm for 2-10 days. The supernatants and pellets were separated by centrifugation at 10,000 rpm (6,060×g) at 4 °C for 15 min. The supernatants were collected to detect extracellular lignocellulolytic enzyme activities.

Carboxymethyl cellulase (CMCase) activity

CMCase activity was assayed using the method described by Sadhu *et al*. [18]. The enzyme solution 0.5 mL was mixed with 0.5 mL of 1.0 % CMC in 50 mM sodium acetate buffer, pH 4.8 and incubated on a shaker at 150 rpm at 50 °C for 30 min. The reaction was stopped by adding 1.0 mL 3,5-dinitrosalicylic acid (DNS) reagent (DNS, 10 g; NaOH, 16 g; potassium sodium tartrate, 300 g and distilled water up to 1 L). The mixture was boiled for 10 min, cooled in ice and its optical density at 540 nm was determined for reducing sugar production [19]. One unit of CMCase activity was defined as the amount of enzyme that released 1 μmol of reducing sugars as glucose per mL enzyme per min under the assay conditions.

Avicellulase activity

The activity of avicellulase was measured using the method described by Sadhu *et al*. [18]. The method to measure avicellulase was similar to CMCase only substituting microcrystalline cellulose (1.0 % Avicel) for CMC. One unit of avicellulase activity was defined as the amount of enzyme that released 1 μmol of reducing sugars as glucose per mL enzyme per min under the assay conditions.

β-Glucosidase activity

The activity of β-glucosidase was measured using the method described by Lemos *et al.* [20]. This was like the CMCase assay, using 0.5 % cellobiose instead of CMC. One unit of β-glucosidase activity was defined as the amount of enzyme that released 1 μmol of reducing sugars as glucose per mL enzyme per min under the assay conditions.

Xylanase activity

The xylanase activity was determined using the same method as a cellulase assay but oat spelt xylan (1.0 % (w/v) was used as a substrate [20]. One unit of xylanase activity was defined as the amount of enzyme that released 1 μmol of reducing sugars as xylose per mL enzyme per min under the assay conditions.

Laccase activity

The laccase activity was determined based on the oxidation of the ABTS [21]. The reaction mixture contained 500 µl of ABTS (1 mM) in 100 mM sodium acetate buffer (pH 5.0), 500 µL of enzyme solution and 1.0 mL of distilled water. The rate of ABTS oxidation was measured spectrophotometrically at 436 nm for 3 min at 27 °C. One unit of laccase activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol ABTS per mL enzyme per min.

Manganese peroxidase activity

The manganese peroxidase (MnP) activity was measured by the oxidation of 2,6-dimethoxyphenol [22]. The reaction mixture contained 250 µl of sodium acetate buffer (100 mM, pH 4.5), 500 µl of 2,6-dimethoxyphenol (0.5 mM), 250 µl of manganese sulfate (1 mM), 500 µl of crude enzyme solution and 750 µl of distilled water. The mixture was incubated at 30 °C and the reaction was initiated by adding 50 µl H_2O_2 (1 mM). The rate of dimethoxyphenol oxidation was determined spectrophotometrically at 469 nm. The absorbance was measured in 1 min interval after addition of hydrogen peroxide. One unit of MnP activity was defined as the amount of enzyme catalyzing the oxidation of 1 µmol of dimethoxyphenol per mL enzyme per min.

Identification of the isolated microorganisms

Twelve microbial isolates from secondary screening step that showed high cellulolytic activities were identified by ribosomal DNA sequencing. Firstly, the microbial genomic DNA was extracted by using the E.Z.N.A® Tissue DNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) according to the manufacturer's recommendations. The 16S rDNA was PCR amplified using the 8F (5′- AGA GTT TGA TCC TGG CTC AG -3′), and 1492R (5′- GGT TAC CTT GTT ACG ACT T -3′) primers for bacteria and actinomyces [23]. Amplifications by PCR were performed with an initial denaturation at 94 °C for 4 min and followed by 20 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and

extension at 72 °C for 45 s followed by final extension at 72 °C for 8 min, and end at 4 °C. For fungi, the 18S rDNA was amplified from the genomic DNA with NS1 (5′- GTA GTC ATA TGC TTG TCT C-3′) and EF3 (5′-TCC TCT AAA TGA CCA AGT TTG -3′) primers [24]. Amplifications by PCR were performed with an initial denaturation at 95 °C for 5 min and followed by 32 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min followed by final extension at 72 °C for 10 min, and end at 4 °C. The PCR products were confirmed by agarose gel electrophoresis in a 1 % agarose gel and were purified with the HiYield™ Gel/PCR DNA Fragments Extraction Kit (Real Biotech Co., Taipei County, Taiwan). The purified PCR products were sequenced by BioDesign. Co., Ltd., Thailand. The percent similarity of the resulting DNA sequences was analyzed through NCBI blast tool on www.ncbi.nlm.nih.gov/blast.

Time courses of lignocellulolytic enzyme productions by the selected isolates

The selected isolates that showed high CMCase, xylanase, laccase and MnP activities were cultivated in 100 mL of LBM broth supplemented with 10 % of decomposed rubber bark as a carbon source in 250 mL Erlenmeyer flasks. Each flask was inoculated with 10 mL of 10^8 CFU/mL for bacteria and actinomyces and 10^8 spores/mL for fungi. All cultures were incubated at 30 °C and at 45 °C with shaking at 150 rpm. After 2 days for bacteria, 6 days for actinomyces and 10 days for fungi, the sample was taken from each culture flask and centrifuged at 10,000 rpm (6,060×g) and 4 °C for 10 min. The supernatants were collected to determined cellulase, xylanase, laccase and MnP activities. The precipitates were used for determination of cell dry weight at 105 °C for 24 h and compared with control.

Statistics

All experiments were carried out in triplicate. Analysis of variance (ANOVA) was performed and means comparisons were run by Duncan's multiple range test (DMRT) at 95 % significant levels. The analysis was performed using the SPSS package (SPSS 16.0 for windows, SPSS Inc, Chicago, IL, USA).

Results and discussion

The characteristics and chemical compositions of the fresh rubber bark were compared to the decomposed rubber bark (**Table 1**). The decomposed rubber bark contained less cellulose, hemicellulose, lignin and organic matter than the fresh rubber bark. The decreasing level of organic matter was related to its mineralization, indicated by the increase in ash content of the decomposed rubber bark (6.29 to 46.04 %). The diminishable organic matter contents may derive from decomposing of lignocelluloses by lignocellulolytic microorganisms which produced cellulase, hemicellulase and ligninase enzymes [25]. The contents of nitrogen (N) (0.92 %), phosphorus (P) (0.43 %) and potassium (K) (0.33 %) of the decomposed rubber bark were low compared to the properties of organic fertilizer in Thailand as N > 1.0 %, P > 0.5 % and K > 0.5 % [26]. Therefore, the composting of rubber bark must be supplemented with the other agro-industrial residues which have rich values of N, P and K. So, the other agro-industrial residues in Thailand were collected and analyzed the chemical compositions. The results reviled that shrimp head had the highest nitrogen content at 8.48 %, lutoid exhibited the highest phosphorus content at 31.07 % while activated sludge presented the highest potassium content at 1.37 % as shown in **Table 2**.

Table 1 Characteristics and chemical compositions of fresh and decomposed rubber bark.

Constituent	Composition of rubber bark (%)	
	Fresh	Decomposed
Moisture	10.77 ± 0.03	6.66 ± 0.02
Cellulose	33.70 ± 0.24	6.09 ± 0.24
Hemicellulose	11.69 ± 0.20	5.25 ± 0.20
Lignin	19.39 ± 0.43	9.17 ± 0.11
Total nitrogen	1.64 ± 0.08	0.92 ± 0.03
Phosphorus-P_2O_5	0.84 ± 0.08	0.43 ± 0.03
Potassium-K_2O	0.58 ± 0.08	0.33 ± 0.02
Total organic carbon	54.36 ± 0.16	26.71 ± 0.24
Organic matter	93.71 ± 0.28	53.96 ± 0.42
Ash	6.29 ± 0.28	46.04 ± 0.42

Percentage (%) was based on dry weight

Table 2 The chemical compositions of agricultural and agro-industrial residues.

Parameters	Composition (% in dry matter)					
	Lutoid	Activated sludge	Shrimp head	Rice bran	Bat guano	Grease
Moisture	39.90 ± 0.01	84.55 ± 0.01	73.24 ± 0.14	9.80 ± 0.04	12.41 ± 0.18	43.30 ± 0.02
Total nitrogen	3.42 ± 0.06	7.13 ± 0.20	8.48 ± 0.15	2.05 ± 0.02	4.08 ± 0.23	1.12 ± 0.07
Phosphorus-P_2O_5	31.07 ± 0.06	4.60 ± 0.06	2.94 ± 0.04	2.94 ± 0.06	3.74 ± 0.03	0.20 ± 0.02
Potassium-K_2O	1.29 ± 0.01	1.37 ± 0.00	0.87 ± 0.01	1.20 ± 0.01	0.54 ± 0.01	0.04 ± 0.00
Total organic carbon	34.94 ± 0.02	43.35 ± 0.05	44.59 ± 0.10	51.86 ± 0.01	24.89 ± 0.09	53.30 ± 0.09
Organic matter	62.89 ± 0.03	78.04 ± 0.09	80.26 ± 0.18	93.34 ± 0.02	44.80 ± 0.16	95.94 ± 0.16
Ash	37.11 ± 0.03	21.96 ± 0.09	19.74 ± 0.18	6.66 ± 0.02	55.21 ± 0.16	4.06 ± 0.16

Isolation of bacteria, actinomyces and fungi from agricultural and agro-industrial residues

A total of 207 microbial colonies were isolated from 4 different sources of agricultural residues including the composting piles, the piles of rubber bark, the cow manure and the soil from the naturally grown bamboo. One hundred and twenty nine isolates could grow at 30 °C {69 isolates were bacteria (B), 45 isolates were actinomyces (A) and 15 isolates were fungi (F)} and 78 isolates could grow at 45 °C (29 isolates were bacteria, 43 isolates were actinomyces and 6 isolates were fungi).

Primary screening of lignocellulolytic microorganisms

All microbial isolates were spotted onto CBM, XBM, ABTS and DMP agar plates for primary screening, to evaluate the lignocellulolytic properties including cellulase, xylanase, laccase and manganese peroxidase activities at 30 °C and 45 °C.

Screening of cellulolytic microorganisms

Among the 207 isolates, only 85 isolates produced clear zones on CBM agar after incubation at 30 °C (40 isolates, **Table 3**) and 45 °C (45 isolates, **Table 4**). The production of a clear zone on CBM agar plates demonstrated that they produced extracellular endoglucanase enzyme which was responsible for CMC hydrolysis [4]. Eighty-five isolates grew well on the medium containing CMC and showed potency indexes ranging from 1.0 to 15.0 and 2.0 to 20.0 at 30 °C and 45 °C, respectively. The isolates B10 from bacteria, A2 from actinomyces and F6 from fungi presented high potency index of cellulase of 14.58, 13.18 and 6.18, respectively, compared with the other isolates after incubation at 30 °C. While

isolates B29 from bacteria, A15 from actinomyces and F14 from fungi showed high potency index of cellulase of 20.13, 20.20 and 8.98, respectively, compared with the others isolates after incubation at 45 °C (**Table 3**).

Table 3 The ability of isolated microorganisms to degrade cellulose, hemicellulose and lignin on media containing CMC, xylan, ABTS and DMP after 2 days of incubation at 30 °C.

Isolate	Potency index			
	CMC	xylan	ABTS	DMP
B1	3.02 ± 0.04^{i}	2.12 ± 0.11^{j}	-	-
B2	9.95 ± 0.07^{g}	5.58 ± 0.11^{h}	-	-
B3	11.02 ± 0.04^{d}	7.04 ± 0.09^{f}	-	-
B4	4.60 ± 0.14^{h}	-	-	-
B5	7.06 ± 0.08^{g}	7.94 ± 0.09^{cd}	-	-
B6	3.08 ± 0.05^{i}	-	-	-
B7	6.99 ± 0.14^{g}	12.00 ± 0.03^{a}	-	-
B8	1.95 ± 0.07^{j}	-	-	-
B9	2.02 ± 0.11^{j}	-	-	-
B10	14.58 ± 0.11^{a}	12.01 ± 0.29^{a}	-	-
B11	14.02 ± 0.04^{a}	4.00 ± 0.17^{i}	-	-
B12	11.00 ± 0.14^{d}	8.02 ± 0.04^{c}	-	-
B13	8.02 ± 0.11^{f}	6.10 ± 0.06^{g}	-	-
B14	11.00 ± 0.01^{d}	9.04 ± 0.06^{b}	-	-
B15	13.02 ± 0.04^{b}	9.02 ± 0.09^{b}	-	-
B16	10.40 ± 0.57^{de}	7.68 ± 0.25^{d}	-	-
B17	11.15 ± 0.07^{d}	7.66 ± 0.15^{e}	-	-
B18	11.98 ± 0.04^{c}	5.93 ± 0.11^{g}	-	-
B19	9.98 ± 0.11^{e}	8.06 ± 0.02^{c}	-	-
B20	7.02 ± 0.04^{g}	4.98 ± 0.04^{i}	-	-
A1	5.60 ± 0.14^{f}	13.02 ± 0.12^{a}	-	-
A2	13.18 ± 0.11^{a}	12.05 ± 0.21^{b}	-	-
A3	7.00 ± 0.14^{e}	5.10 ± 0.07^{f}	-	-
A4	8.08 ± 0.05^{d}	10.12 ± 0.04^{e}	-	-
A5	12.07 ± 0.10^{c}	11.10 ± 0.14^{d}	-	-
A6	11.99 ± 0.01^{c}	10.10 ± 0.09^{e}	-	-
A7	12.08 ± 0.11^{c}	11.08 ± 0.04^{d}	-	-
A8	12.60 ± 0.14^{b}	11.48 ± 0.04^{c}	-	-
A9	12.05 ± 0.07^{c}	11.45 ± 0.14^{c}	-	-
F1	3.02 ± 0.11^{e}	-	-	-
F2	6.06 ± 0.09^{a}	-	7.10 ± 0.14^{a}	7.07 ± 0.10^{a}
F3	5.44 ± 0.06^{b}	-	-	-
F4	5.02 ± 0.32^{c}	5.02 ± 0.04^{d}	-	-
F5	5.00 ± 0.28^{c}	5.51 ± 0.16^{c}	-	-
F6	6.18 ± 0.25^{a}	6.50 ± 0.14^{a}	5.64 ± 0.18^{b}	5.44 ± 0.06^{b}
F7	4.50 ± 0.14^{d}	3.90 ± 0.14^{e}	1.49 ± 0.16^{c}	
F8	5.07 ± 0.10^{bc}	5.94 ± 0.08^{b}	1.69 ± 0.13^{c}	1.53 ± 0.10^{c}
F9	4.30 ± 0.14^{d}	-	-	-
F10	1.10 ± 0.14^{f}	3.74 ± 0.20^{e}	-	-
F11	4.85 ± 0.16^{c}	5.52 ± 0.11^{c}	5.74 ± 0.21^{b}	5.32 ± 0.18^{b}

*B = bacteria, A = actinomyces and F = fungi

Table 4 The ability of isolated microorganisms to degrade cellulose, hemicellulose and lignin on media containing CMC, xylan, ABTS and DMP after 2 days of incubation at 45 °C.

Isolate	Potency index			
	CMC	xylan	ABTS	DMP
B21	2.88 ± 0.18^{m}	-	-	-
B22	7.00 ± 0.14^{i}	2.58 ± 0.11^{p}	-	-
B23	17.12 ± 0.11^{c}	8.53 ± 0.11^{e}	-	-
B24	2.98 ± 0.11^{m}	3.03 ± 0.18^{no}	-	-
B25	15.98 ± 0.04^{d}	6.23 ± 0.11^{i}	-	-
B26	16.04 ± 0.23^{d}	6.10 ± 0.14^{i}	-	-
B27	19.99 ± 0.01^{a}	2.78 ± 0.18^{mn}	-	-
B28	20.06 ± 0.21^{a}	3.82 ± 0.18^{kl}	-	-
B29	20.13 ± 0.04^{a}	10.15 ± 0.21^{c}	-	-
B30	4.88 ± 0.04^{k}	4.95 ± 0.14^{j}	-	-
B31	14.00 ± 0.28^{e}	14.92 ± 0.11^{b}	-	-
B32	17.93 ± 0.11^{b}	6.80 ± 0.14^{h}	-	-
B33	15.95 ± 0.21^{d}	4.42 ± 0.18^{k}	-	-
B34	15.90 ± 0.14^{d}	6.10 ± 0.07^{i}	-	-
B35	8.90 ± 0.14^{h}	7.15 ± 0.14^{g}	-	-
B36	18.05 ± 0.07^{b}	6.85 ± 0.21^{h}	-	-
B37	17.02 ± 0.18^{c}	9.68 ± 0.25^{d}	-	-
B38	11.92 ± 0.18^{f}	7.90 ± 0.14^{f}	-	-
B39	9.08 ± 0.11^{h}	2.95 ± 0.21^{o}	-	-
B40	11.02 ± 0.11^{g}	15.12 ± 0.18^{a}	-	-
B41	3.95 ± 0.21^{l}	3.98 ± 0.25^{l}	-	-
B42	11.98 ± 0.07^{f}	3.50 ± 0.07^{m}	-	-
B43	13.93 ± 0.11^{e}	3.10 ± 0.14^{n}	-	-
B44	5.95 ± 0.14^{j}	4.42 ± 0.18^{k}	-	-
B45	3.98 ± 0.25^{l}	2.10 ± 0.07^{q}	-	-
A10	10.12 ± 0.18^{h}	-	-	-
A11	14.08 ± 0.11^{e}	18.05 ± 0.14^{c}	-	-
A12	1.98 ± 0.04^{l}	4.92 ± 0.11^{k}	-	-
A13	19.20 ± 0.07^{b}	17.08 ± 0.11^{e}	-	-
A14	17.18 ± 0.18^{c}	16.05 ± 0.14^{f}	-	-
A15	20.20 ± 0.21^{a}	21.15 ± 0.14^{b}	-	-
A16	9.02 ± 0.21^{i}	11.98 ± 0.11^{i}	-	-
A17	8.00 ± 0.11^{j}	11.90 ± 0.07^{i}	-	-
A18	17.05 ± 0.14^{c}	17.25 ± 0.14^{d}	-	-
A19	7.10 ± 0.14^{k}	11.18 ± 0.04^{j}	-	-
A20	9.12 ± 0.11^{i}	13.02 ± 0.11^{h}	-	-
A21	20.18 ± 0.04^{a}	21.92 ± 0.11^{a}	-	-
A22	10.98 ± 0.11^{g}	13.12 ± 0.11^{h}	-	-
A23	12.00 ± 0.21^{f}	13.12 ± 0.04^{h}	-	-
A24	15.22 ± 0.11^{d}	15.22 ± 0.11^{g}	-	-
A25	12.18 ± 0.04^{f}	16.92 ± 0.11^{e}	-	-
F12	8.08 ± 0.18^{b}	8.00 ± 0.07^{a}	4.32 ± 0.04^{b}	3.60 ± 0.07^{b}
F13	7.32 ± 0.18^{c}	-	-	-
F14	8.98 ± 0.04^{a}	8.02 ± 0.11^{a}	-	-
F15	6.92 ± 0.18^{d}	6.12 ± 0.11^{b}	6.15 ± 0.14^{a}	7.18 ± 0.18^{a}

*B = bacteria, A = actinomyces and F = fungi

Screening of xylanolytic microorganisms

From the total 207 isolates, 74 isolates showed the ability to produce xylanase on XMB agar when the plates were incubated at 30 °C (32 isolates, **Table 3**) and at 45 °C (42 isolates, **Table 4**). Isolates B10 from bacteria, A1 from actinomyces and F6 from fungi exhibited the highest potency index of 12.01, 13.02 and 6.50, respectively, compared with the other isolates when incubated at 30 °C. In contrast, isolates B40 from bacteria, A21 from actinomyces and F14 from fungi presented the highest potency index of 15.12, 21.92 and 8.02, respectively, when incubated at 45 °C.

Screening of ligninolytic microorganisms

All isolated strains were cultured on ABTS and DMP agar plates and incubated at 30 °C and 45°C. The formation of the dark-green on ABTS and the blue colored on DMP indicated a positive extracellular oxidoreductase secretion [27]. The diameter of the color intensity was used to monitor the level of ligninolytic enzyme production of each strain. The result demonstrated that only a few fungi could produce laccase (ABTS agar) and manganese peroxidase (DMP agar). The isolates F2 showed the highest potency index of 7.10 and 7.07 on ABST and DMP agar, respectively, when incubated at 30 °C. While, F15 was identified as the best ligninolytic culture based on the color change on the ABTS agar (potency index 6.15) and DMP agar (potency index 7.18) when incubated at 45 °C. (**Tables 3** and **4**).

The agar assay screenings were used extensively for primary of linocellulolytic screening microorganisms. In a few studies, researchers have examined the cellulolytic enzymes, FPase, CMCase, β-glucosidase, which screened from *Aspergillus* sp. and *Penicillium* sp. The results revealed that these 2 microorganisms secreted cellulolytic enzymes which showed a clear zone on selective media [28]. Also, some bacteria could produce xylanase and exhibited a clearing zone on the xylanolytic screening agar [29]. Aside from that Kumari *et al*. [30] suggested that the screening of lignin degrading enzymes on ABTS agar was dependent on the development of that color. The darker the color stronger the oxidation of lignin.

Secondary screening of lignocellulolytic microorganisms

Twelve isolates that showed a high potency index on the selective agar were examined for lignocellulolytic enzyme production in the LBM broth supplemented with 10 % of the decomposed rubber bark as a carbon source. Only 12 isolates namely B10, B15, A1, A2, F6, F11 (incubated at 30 °C) and B29, B37, A15, A21, F12, F15 (incubated at 45 °C) showed high lignocellulolytic enzyme productions.

The isolates B15, A2 and F6 showed the highest lignocellulolytic enzyme activities when grown in the decomposed rubber bark-LBM broth at 30 °C (**Table 5**). While, isolates B37, A21 and F12 showed the highest enzyme activities when grown in the medium at 45 °C (**Table 6**). It is noted that only fungal isolates could produce ligninolytic enzymes (laccase and MnP) which is consistent with the primary screening. Moreover, the fungal isolate F6 incubated at 30 °C and F12 incubated at 45 °C exhibited the highest activities of all enzymes compared with the isolates from bacteria and actinomyces. For isolate F6, it produced enzyme activities at 0.61, 0.34, 0.56, 0.61, 0.81 and 0.56 U/mL of CMCase, avicellulase, β-glucosidase, xylanase, laccase and MnP, respectively. While, isolate F12 produced enzyme activities at 0.56, 0.28, 0.46, 0.48, 0.54 and 0.33 U/mL of CMCase, avicellulase, β-glucosidase, xylanase, laccase and MnP, respectively. However, it is hard to conclude that among bacteria, actinomyces and fungi which one are the best microorganisms that can produce ligninolytic enzymes. Nevertheless, the microorganisms isolated in this study (B15, B37, A2, A21, F6 and F12) showed higher lignocellulolytic enzyme activities than the isolates obtained by Samuel *et al*. [31] and Gautam *et al*. [32]. Samuel *et al*. [31] isolated 4 bacterial isolates (*Bacillus* sp., 2 *Pseudomonas* sp. and *Proteus* sp.) and 2 fungal strains (*Aspergillus niger* and *A. fumigatus*) by spread plate technique using coir waste and sawdust as a substrate. The bacteria strain, *Bacillus* sp., showed the highest cellulase activity of 0.09 and 0.08 U/mL with coir waste and sawdust as a substrate, respectively. Fungal strain, *A. niger*, showed higher cellulase activity of 0.1 and 0.1 U/mL with coir waste and sawdust as a substrate, respectively. Gautam *et al*. [32] isolated 85 bacterial and 165 fungal isolates from municipal solid waste, compost, and

soil samples collected from different areas of Jabalpur. Among these, *Trichoderma viride* showed the maximum exoglucanase, endoglucanase and β-glucosidase activities as 2.22, 2.03 and 1.98 U/mL, respectively after 6 days of incubation at 30 °C on the basal salt medium containing 1 % cellulose as a sole carbon source.

After the secondary screening, 4 isolates each from bacteria, actinomyces and fungi which showed the high level of linocellulolytic were subjected to species identification. In the meantime, the 6 isolates previously mentioned (B15, B37, A2, A21, F6 and F12) were selected for the preparation of inoculums in compost production.

Table 5 Lignocellulolytic enzymes of selected microorganisms in LBM broth containing the decomposed rubber bark as a carbon source at 30 °C.

Isolate	Lignocellulolytic enzyme activities (Unit/mL)					
	CMCase	Avicellulase	β-glucosidase	Xylanase	Laccase	MnP
B10	0.17 ± 0.02^{e}	0.12 ± 0.03^{e}	0.26 ± 0.01^{d}	0.22 ± 0.01^{d}	-	-
B15	0.21 ± 0.01^{d}	0.17 ± 0.01^{d}	0.33 ± 0.01^{c}	0.32 ± 0.02^{c}	-	-
A1	0.31 ± 0.02^{c}	0.28 ± 0.02^{b}	0.32 ± 0.02^{c}	0.30 ± 0.01^{c}	-	-
A2	0.33 ± 0.01^{c}	0.25 ± 0.01^{c}	0.39 ± 0.02^{b}	0.36 ± 0.02^{b}	-	-
F6	0.61 ± 0.02^{a}	0.34 ± 0.02^{a}	0.56 ± 0.02^{a}	0.61 ± 0.02^{a}	0.81 ± 0.02	0.56 ± 0.02
F11	0.56 ± 0.01^{b}	0.31 ± 0.02^{ab}	0.54 ± 0.01^{a}	0.39 ± 0.01^{b}	0.72 ± 0.03	0.42 ± 0.02

B10 = *Bacillus licheniformis*, B15 = *B. subtilis*, A1 = *Streptomyces mexicanus*, A2 = *S. albogriseolus*, F6 = *Trichoderma asperellum*, F11 = *Pycnoporus coccineus*

Table 6 Lignocellulolytic enzymes of selected microorganisms in LBM broth containing the decomposed rubber bark as a carbon source at 45 °C.

Isolate	Lignocellulolytic enzyme activities (Unit/mL)					
	CMCase	Avicellulase	β-glucosidase	Xylanase	Laccase	MnP
B29	0.12 ± 0.02^{e}	0.09 ± 0.01^{b}	0.11 ± 0.01^{e}	0.13 ± 0.01^{e}	-	-
B37	0.16 ± 0.01^{d}	0.11 ± 0.01^{ab}	0.14 ± 0.01^{d}	0.22 ± 0.02^{c}	-	-
A15	0.25 ± 0.01^{c}	0.18 ± 0.02^{ab}	0.23 ± 0.02^{c}	0.18 ± 0.01^{d}	-	-
A21	0.28 ± 0.01^{c}	0.15 ± 0.02^{ab}	0.26 ± 0.02^{b}	0.19 ± 0.02^{d}	-	-
F12	0.56 ± 0.01^{a}	0.28 ± 0.02^{a}	0.46 ± 0.01^{a}	0.48 ± 0.01^{a}	0.54 ± 0.01	0.33 ± 0.02
F15	0.44 ± 0.02^{b}	0.21 ± 0.01^{ab}	0.44 ± 0.02^{a}	0.29 ± 0.02^{b}	0.32 ± 0.02	0.24 ± 0.03

B29 = *Bacillus amyloliquefaciens*, B37 = *B. tequilensis*, A15 = *Laceyella sacchari*, A21 = *S. thermocarboxydus*, F12 = *Thermoascus aurantiacus*, F15 = *Chaetomium globosum*

Identification of the isolated microorganisms

Identification of the isolated microorganisms producing lignocellulolytic enzymes from agricultural and agro-industrial residues was shown in **Table 7**. The isolated bacteria B10, B15, B29, B37 and actinomyces A1, A2, A15 and A21 were identified by 16S rDNA gene sequencing analysis. All 4 bacterial isolates were *Bacillus*. The B10 and B15 isolates showed 99 % similarity to *Bacillus licheniformis* and *B. subtilis*, respectively. The B29 and B37 isolates showed 100 % similarity to *B. amyloliquefaciens* and *B. tequilensis*, respectively. The actinomyces isolates A1 and A2 showed 99 % similarity to *Streptomyces mexicanus* and *S. albogriseolus*, respectively. The A15 isolate showed 100 % similarity to *Laceyella sacchari* and the A21 isolate had 99 % similarity to *S. thermocarboxydus*. All the isolated bacterial strains were classified as bacteria in the biosafety level 1.

The isolated fungi F6, F11, F12 and F15 were identified by 18S rDNA gene sequencing analysis. The F6 isolate was 100 % similar to *Trichoderma asperellum*. The F11, F12 and F15 isolates were 99 % similar to *Pycnoporus coccineus*, *Thermoascus aurantiacus* and *Chaetomium globosum*, respectively. All the fungal strains were classified as fungi in the biosafety level 1. The rDNA sequences of these 12 isolates were deposited in the GenBank with the accession numbers as shown in **Table 7**. Aside from our study, Devi and Kumar [33] also screened 70 fungal isolates from different animal dung manure soil to select the strains that can produce cellulase and xylanase. The data revealed that 10 isolates were found to be capable of cellulase and xylanase production. Among them, 2 isolates were identified as *Aspergillus* sp. and 8 isolates were *Trichoderma* sp. Taboa and Monsalud [34] isolated 225 bacterial isolates from several mangrove areas in the Philippines and found that 154 isolates had the ability to produce cellulase. Five out of 154 isolates showed strong cellulase activity and were identified as *Cellulomonas* sp., *Bacillus* sp., *B. cereus*, *B. licheniformis*, and *B. pumilus.*

Table 7 Identification of lignocellulolytic enzyme-producing microorganisms.

Isolates	Strains	% homology	GenBank accession no.
B10	*Bacillus licheniformis*	99	LC133185
B15	*Bacillus subtilis*	99	LC133139
B29	*Bacillus amyloliquefaciens*	100	LC133346
B37	*Bacillus tequilensis*	100	LC133347
A1	*Streptomyces mexicanus*	99	LC133348
A2	*Streptomyces albogriseolus*	99	LC152199
A15	*Laceyella sacchari*	100	LC133349
A21	*Streptomyces thermocarboxydus*	99	LC152202
F6	*Trichoderma asperellum*	100	LC152196
F11	*Pycnoporus coccineus*	99	LC133350
F12	*Thermoascus aurantiacus*	99	LC152198
F15	*Chaetomium globosum*	99	LC133351

Time courses of lignocellulolytic enzyme productions by selected isolates

CMCase and xylanase productions by the selected microorganisms are shown in **Figures 1-3**. *B. subtilis* B15 and *B. tequilensis* B37 which grew rapidly in LBM broth supplemented with 10 % decomposed rubber bark from 2 to 16 h and then entered the stationary phase and produced the highest CMCase (0.36 and 0.28 U/mL, respectively) and xylanase (0.52 and 0.42 U/mL, respectively) at 16 h of cultivation (**Figure 1**). The contrast with CMCase and xylanase produced by *B. subtilis* strains SL9-9 which cultured in CMC liquid medium that showed a maximum CMCase and xylanase production after 72 and 96 h of cultivation [35]. Also, Seo *et al*. [36] reported that *B. licheniformis* was grown in a mineral salts medium containing 1 % (w/v) of wheat bran producing a maximum of CMCase and xylanase at 48 h of incubation.

The isolates *S. albogriseolus* A2 and *S. thermocarboxydus* A21 which were operantly inoculated in LBM broth supplemented with 10 % of decomposed rubber bark entered the stationary phase and produced the highest enzyme activities at 96 h of cultivation. *S. albogriseolus* A2 produced CMCase and xylanase at 0.58 and 072 U/mL, respectively, while *S. thermocarboxydus* A21 produced CMCase and xylanase at 0.52 and 0.46 U/mL, respectively (**Figure 2**). If comparing *S. albogriseolus* A2 and *S. thermocarboxydus* A21 to other actinomyces strains, *S. albogriseolus* subp. *cellulolyticus* NEAE-J showed that the maximum CMCase and xylanase production in basal salt medium containing 1 % (w/v) of sugarcane bagasse occurred after 144 h of incubation [37]. The maximum xylanase production of the *S. thermocarboxydus* strain MW8 in SNX medium containing 0.5 % (w/v) birchwood xylan occurred at 96 h [38].

For fungi, *T. asperellum* F6 and *Thermoascus aurantiacus* F12 entered the stationary phase and exhibited the highest activities of CMCase (0.78 and 0.68 U/mL, respectively) and xylanase (0.90 and 0.61 U/mL, respectively) at 144 h of cultivation (**Figure 3**) but produced the highest activities of laccase (1.32 and 0.99 U/mL, respectively) and MnP (1.16 and 0.81 U/mL, respectively) at 192 h of cultivation (**Figure 3**). Selvamani *et al*. [39] reported that *T. asperellum* SD1 was cultured in glucose minimum medium produced the maximum of laccase and lignin peroxidase (LiP) after 48 h. On the other hand, MnP was produced after 144 h. Dar and Phutela [40] reported that *T. aurantiacus* MTCC 375 showed the maximum activity of laccase, MnP and LiP in Mandel's medium using 5 g paddy straw as a substrate under solid state fermentation after 192, 96 and 144 h, respectively. *T. aurantiacus* ATCC 204492 produced the maximum activity of CMCase and xylanase using 15 g sugarcane bagasse and 2 % (v/v) Vogel solution at 240 h for both enzymes [41]. The results obtained indicated that the enzyme production related to the growth of microorganisms and maximum enzyme productions were found during the stationary phase.

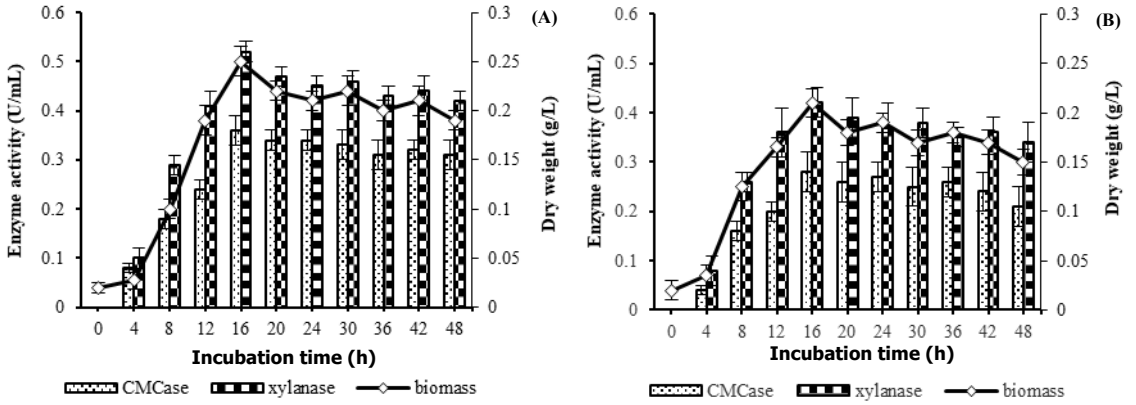

Figure 1 Time course for biomass, CMCase and xylanase productions in LBM broth supplemented with 10 % decomposed rubber bark by *Bacillus subtilis* B15 at 30 °C (A) and *B. tequilensis* B37 at 45 °C (B).

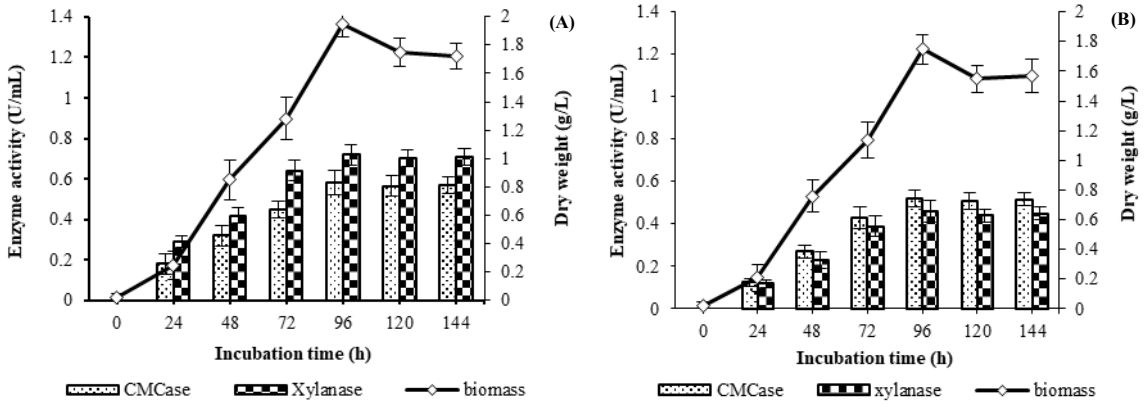

Figure 2 Time course for biomass, CMCase and xylanase productions in LBM broth supplemented with 10 % decomposed rubber bark by *Streptomyces albogriseolus* A2 at 30 °C (a) and *S. thermocarboxydus* A21 at 45 °C (b).

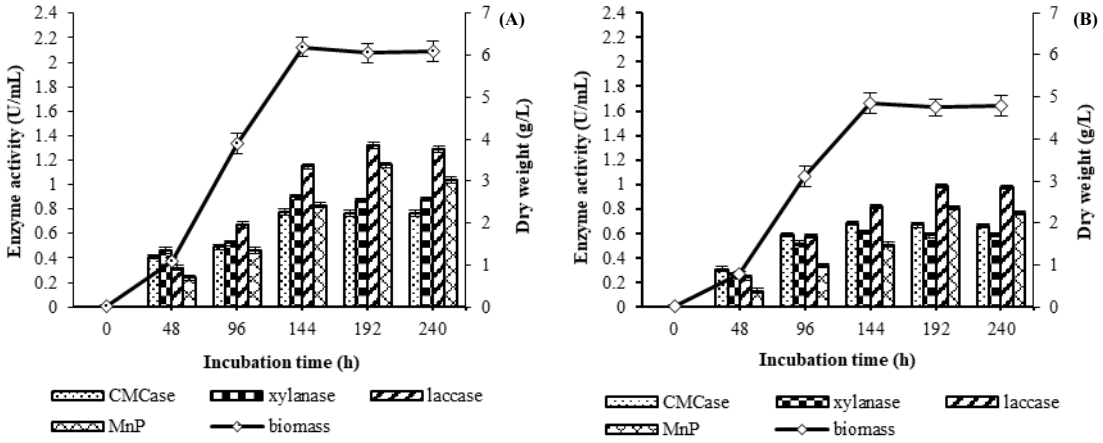

Figure 3 Time course for biomass, CMCase, xylanase, laccase and MnP productions in LBM broth supplemented with 10 % decomposed rubber bark by *Trichoderma asperellum* F6 at 30 °C (a) and *Thermoascus aurantiacus* F12 at 45 °C (b).

Conclusions

The bacteria, actinomyces and fungi producing lignocellulolytic enzymes were isolated from decomposed agricultural and agro-industrial residues. Twelve isolates that showed wide clear zones or intense zones were cultured in LBM agar containing decomposed rubber bark as a carbon source. The isolates B15 (*B. subtilis*), A2 (*S. albogriseolus*), F6 (*T. asperellum*) as mesophilic microbes and B37 (*B. tequilensis*), A21 (*S. thermocarboxydus*), and F12 (*Thermoascus aurantiacus*) as thermophilic microbes showed the highest cellulase and xylanase activities. Only the isolate F6 and F12 produced laccase and manganese peroxidase. Therefore, these 6 isolates will be used as inoculums for composting of rubber bark in further research work.

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