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ORIGINAL PAPER

Isolation and screening of potential actinobacteria for rapid composting of rice straw

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Abstract Rice straw is produced as a by-product from rice cultivation, which is composed largely of lignocellulosic materials amenable to general biodegradation. Lignocellulolytic actinobacteria can be used as a potential agent for rapid composting of bulky rice straw. Twenty-five actinobacteria isolates were isolated from various in situ and in vitro rice straw compost sources. Isolates A2, A4, A7, A9 and A24 were selected through enzymatic degradation of starch, cellulose and lignin followed by the screening for their adaptability on rice straw powder amended media. The best adapted isolate (A7) was identified as *Micromonospora carbonacea.* It was able to degrade cellulose, hemicelluloses and carbon significantly ($P \leq 0.05$) over the control. C/N ratio was reduced to 18.1 from an initial value of 29.3 in 6 weeks of composting thus having the potential to be used in large scale composting of rice straw.

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Introduction

Rice, the main cereal crop in the world, is cultivated to more than 148 million hectares under a wide range of ecosystems. In 1960, total rice production was 150 million tons which increased to 645 million tons in 2007. At least 114 countries grow rice and more than 50 countries have an annual production of 100,000 tons or more (USDA 2008). For every ton of harvested grain, about 1.35 tons of rice straw remains in the field which generate huge amount of straw annually (Kadam et al. 2000). The disposal of rice straw is a problem due to the huge bulk material, slow degradation rate and harboring of diseases. Moreover, it cannot be used as animal feed due to its low digestibility, low protein, high lignin and silica content. In many countries including Malaysia, the huge amount of straw is disposed through open-burning, which causes serious environmental problems as well as a threat to public health.

Rice straw is a potential food source for microorganisms like bacteria, fungi and actinobacteria. It could be converted into a valuable end product in a short period of time through microbial composting process. Rice straw compost is most commonly

applied to paddy fields in Japan to improve soil fertility and increase yield (Hatamoto et al. 2008). To make the rice straw composting process economically viable, lignocellulolytic microbes based biodegradation may be an effective alternative to in situ burning (Kumar et al. 2008). The compost can serve as an excellent source of nutrients in organic farming to mitigate the ill-effects of increasing fertilizer use. Actinobacteria are well known for their ability to decompose complex molecules, particularly lignocellulosic components, which make them important agents in composting process (Tang et al. 2007). They can survive in the high temperature during composting and generate soluble carbohydrate from rice straw (Ball et al. 1990). Fungi and bacteria are reported to dominate in the mesophilic phase for the utilization of the soluble and easily metabolized carbohydrates. Whereas actinobacteria gradually replace the mesophilic populations and dominated in the thermophilic phase for the mineralization of most recalcitrant components to simple sugar during the composting of lignocellulosic wastes (Allgaier et al. 2010). Hence, composting of rice straw pre-inoculated with potential lignocellulolytic actinobacteria perhaps play important role in the efficient and rapid composting of bulky rice straw. Thus, the aim of the study was to isolate, select and characterize the potential actinobacteria for rapid biodegradation of rice straw.

Materials and methods

Sample collection for isolation of Actinobacteria

Samples were collected from two main sources: in situ sources or naturally decomposed rice straw and in vitro sources or induced composting of rice straw.

In situ sources

In situ samples were collected from naturally decomposed rice straw, rice straw residues, and soils collected from dairy and goat farms, and rice fields of Universiti Putra Malaysia as well as the rice growing areas in Kuala Selangor, Malaysia. Five cores of samples were randomly taken from 5 cm depth and pooled in a clean plastic bag and stored at 4°C until use.

In vitro sources

In vitro source was collected from the compost of rice straw amended with chicken manure. The composting process was conducted at the Composting Unit, Universiti Putra Malaysia. Rice straw and chicken manure were collected from rice farmer at Kuala Selangor, Malaysia and Smart Agenda Sdn. Bhd. (536356k), Kuala Lumpur, Malaysia, respectively. Rice straw was air-dried to facilitate the grinding process. Straw was ground using a grinding machine (SJ 500) to a maximum particle size of 500 μm. Straw was mixed with chicken manure in a 1:1 ratio (w/w) and water was added to the composting substrates and mixed thoroughly to obtain 60% moisture content. Initial moister content was measured using a moister content meter. Composting materials were placed in a perforated Styrofoam container (50 × 37 × 35 cm) and covered to preserve the heap temperature and moisture. The heaps were turned every other day for the first 3 weeks and thereafter once in a week. Four replications were used and each Styrofoam container represented a replicate. The temperature was recorded daily. Samples were collected weekly.

Isolation of lignocellulolytic actinobacteria

Isolation of lignocellulolytic actinobacteria was done by placing 10 g samples and 90 ml of sterile distilled water into 250 ml Erlenmeyer flasks. The solution was stirred at 150 rpm for an hour. Serial dilutions of 10^{-2}–10^{-5} were prepared by sequentially transferring one ml samples into test tubes containing 9 ml of sterile distilled water. 100 μl samples were pipetted onto actinobacteria selective media (pH 8.1 ± 0.2, Difco^{TM}). The plates were examined regularly. The single colony of actinobacterium was transferred aseptically onto fresh actinobacteria selective media to obtain the pure culture.

Biochemical screening for lignocellulolytic activity

Enzymatic degradation of starch

The ability of actinobacteria isolates to degrade starch was tested using 10% starch amended actinobacteria selective media (pH 7.9 ± 0.2). The solidified medium was inoculated with 3 μl of

actinobacterium suspension (10^8 CFU ml^{-1}) and incubated at 28 ± 2°C for 72 h. The colony growing on the media was flooded with iodine solution (10%) and allowed to be in contact for 30 s. Un-hydrolyzed starch reacts with iodine and turn black and hydrolyzed starch creates halo zone around the colony.

Enzymatic degradation of cellulose

Cellulose degradation was tested on Jensen's media (20.0 g sucrose, 1.0 g K_2HPO_4, 0.1 g $FeSO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g NaCl, 0.005 g $NaMoO_4$, 2.0 g $CaCO_3$, 15.0 g agar, 2.0 g carboxymethyl cellulose and 1.0 l distilled water) with pH 7.5 ± 0.2 of which 20 ml was poured into each Petri plate (Jensen 2008). The solidified medium was inoculated with 3 µl of actinobacterium suspension (108 CFU ml^{-1}) and incubated at 28 ± 2°C for 72 h. Then, the media was flooded with an aqueous solution of Congo red (1.0 mg/ml media) for 15 min. The Congo red solution was poured off and plate was flooded with 1 M NaCl for 15 min. Degradation of cellulose was visualized as a halo zone around the colony. The diameter of the halo zone around the colony was used to assay for the degree of cellulose degradation (Teather and Wood 1982).

Enzymatic degradation of lignin

Lignin degradation was tested using the media proposed by Archibald (1992) with some modifications. The media contained (g/l) $(NH_4)_2HPO_4$ 1.0, KCl 0.2, $MgSO_4 \cdot 7H_2O$ 0.2, yeast extract 2.0, glucose 2.0, azure B (0.01% w/v) 0.1, Agar 15.0 and 1.0 l distilled water (pH 7.5 ± 0.2). All reagents were dissolved in distilled water and autoclaved at 121°C for 20 min and 20 ml was poured into each Petri plate. The solidified medium was inoculated carefully with 3 µl of actinobacterium suspension (10^8 CFU ml^{-1}) and incubated at 28 ± 2°C for 72 h. Lignin degradation was indicated by the growth of the actinobacteria colony on the media. The growth diameter was recorded.

Lignocellulolytic activity of selected actinobacteria in rice straw powder (RSP) amended media

Five selected actinobacteria (A2, A4, A7, A9 and A24) having optimum lignocellulolytic activity based on in vitro screening above were further screened for their adaptability to different percentages (0, 10, 20 and 25%) of RSP amended actinobacteria selective media. The range of the pH was 7.5–8.1 ± 0.2. The cultural media was autoclaved at 121°C for 20 min and 20 ml of media was poured into each Petri dish. The solidified media was inoculated with 3 µl of the actinobacterium suspension (10^8 CFU ml^{-1}) and incubated at 28 ± 2°C for 72 h. Radial growth was recorded daily.

Identification of potential (selected) actinobacteria

The potential isolate (A7) of actinobacterium in term of lignocellulolytic activities and growth on RSP amended media was identified using the BIOLOG identification system (Biolog Inc., 3938, Trust Way, Hayward, CA, USA) with the software Microstation system release version 4.20.

Evaluation of potential isolate (A7) to in vitro composting of rice straw

Isolate (A7) showing the optimum lignocellulolytic activity and best adapted to growth on rice straw powder amended actinobacteria selective media was chosen for in vitro rice straw composting study. Rice straw was ground and sieved through 2 mm filter. Rice straw and chicken manure were mixed in a ratio of 1:1 (w/w) and amended with distilled water to obtain a moisture content of 60% (w/w). The moisture content was adjusted with moisture meter (HH2 Meter, ΔT Delta devices, Cambridge, England). The substrates were inoculated with 10% (v/w) of actinobacterium suspension (A7) with concentration of 10^8 CFU ml (Vargas-Garcia et al. 2007). The amended substrates were transferred into individual plastic bag each containing 150 g of substrate and incubated for 6 weeks. The plastic bags were turned regularly every week to provide aeration. Samples were collected at 3 and 6 weeks during the composting process. Non-inoculated substrates served as control.

Analysis of C/N ratio

During each sampling time the decomposed rice straw was analyzed for C/N ratio. Organic matter was determined as loss of weight on ignition from sample

maintained at 500°C for 4 h (Storer 1984) and total carbon was calculated as 58% of the organic matter Chefetz et al. (1996). The nitrogen content was measured by using the Digestion method (Brainina et al. 2004).

Determination of cellulose, hemicelluloses and lignin

The cellulose, hemicelluloses and lignin content were analyzed using neutral detergent fibre (NDF) and acid detergent fibre (ADF) method (Van Soest et al. 1991).

Experimental design and data analysis

All the experiments were conducted using completely randomized design (CRD) with five replications. The data were subjected to analysis of variance (ANOVA) and tested for significance using Least Significant Difference (LSD) by PC-SAS software (SAS Institute Cary NC 2001). To group the microbial isolates based on their biochemical activities, data were subjected to cluster analysis and a clustering tree was constructed using S-PLUS (Struyf et al. 1997).

Results and discussion

Enzymatic degradation of starch, cellulose and lignin

A total of 25 isolates of actinobacteria were isolated from various in situ and in vitro sources of rice straw compost and assessed for their lignocellulolytic activity on media containing starch, cellulose and azure B (Table 1). Twenty-two isolates were able to produce halo zones on media containing starch after staining with iodine solution (Fig. 1a). Starch, composed of two polymers, amylose and amylopectin, is a biopolymer consisting of α-D-glucose joined together to form large macromolecules. Actinobacteria that showed positive response might produce glucoamylase and α-amylase enzymes on the starch supplemented media. The results are in agreement with Nigam and Singh (1994) who claimed that actinobacteria produce an array of enzymes, glucoamylase, α-amylase and glucose isomerase during starch hydrolysis.

Sixteen actinobacteria isolates formed halo zone on media containing carboxymethyl cellulose (CMC) after staining with Congo red solution (Fig. 1b). Five isolates (A2, A4, A7, A9 and A24) showed relatively higher activity and formed larger zone (>5.00 mm) whereas 11 other isolates formed smaller zone (<5.00 mm). Isolate A7 formed the highest halo zone (9.00 mm) followed by A4, A9, A2 and A24 (8.40, 8.20, 7.80 and 6.18 mm), respectively. The production of halo zones on CMC-media confirmed that they produced extracellular endoglucanase enzyme which was responsible for CMC hydrolysis. McCarthy (1987) reported several actinobacteria strains produced hydrolytic enzymes, extracellular endoglucanase and exoglucanase as well as cell-bound β-glucosidase during growth on substrates containing cellulose.

All isolates grew well on the media containing azure B with colony diameter ranging from 4.02 to 18.98 mm (Fig. 1c). Among the 25 isolates, 8 isolates (A2, A3, A4, A6, A7, A9, A10 and A24) produced relatively larger colony (>6.50 mm). The highest colony was formed by isolate A7 (18.98 mm) followed by A9, A24, A4 and A2 with the colony diameter of 15.96, 15.10, 14.36 and 7.66 mm, respectively. Lignin forms an irregular non-crystalline network in plant cell wall to protect cellulose and hemicelluloses which is highly resistant to biodegradation. Microbes having the ligninolytic enzyme systems can degrade lignin to CO_2 (Martin Hofrichter 2002). Actinobacteria cleaved lignin barrier through diffusible chemical process by utilizing the ligninolytic enzymes, lignin peroxidases and phenol oxidases (Li et al. 2009). The actinobacteria isolates grown on azure B media revealed their potential of producing lignin peroxidase (LiP) (Archibald 1992; Pangallo et al. 2009). Ramachandran et al. (2000) also found LiP in actinobacteria and observed increased activity in the presence of H_2O_2. Crawford (1978) reported that lignin from actinobacteria decayed softwood was extensively demethylated, distorted aromatic rings and undergone side-chain oxidation which were suggested to produce extracellular ligninolytic enzymes for breaking lignin barrier and exposed cellulose and hemicelluloses for further biodegradation. However, azure B method is not totally reliable indicator for the presence of LiP enzyme in an actinobacterium as LiP assay is a complex process. Therefore, all the selected actinobacteria were further screened against rice straw powder (RSP) to confirm their lignocellulolytic activity.

The biochemical performances towards lignocelluloses of 25-isolates were combined in a single cluster analysis producing a dendrogram derived

Table 1 The ability of actinobacteria to degrade starch, cellulose and lignin tested on media containing starch, carboxymethyl cellulose and azure B

Isolates no	Source of isolates	Starch		*Cellulose halo zone (mm)	*Lignin colony growth (mm)
		Positive (+)	Negative (−)		
	In situ				
A1	Dairy farm	+		0.00g	5.09h
A2	Dairy farm	+		7.80b	7.32d
A3	Dairy farm	+		2.40f	7.66d
A4	Dairy farm	+		8.40ab	14.36c
A5	Goat farm	+		0.00g	4.62hij
A6	Goat farm	+		4.00d	6.96de
A7	Goat farm	+		9.00a	18.98a
A8	Rice straw compost	+		0.00g	4.02j
A9	Rice straw compost	+		8.20b	15.96b
A10	Rice field		−	0.00g	6.94de
A11	Rice field	+		0.00g	5.90fg
A12	Rice field	+		2.40f	4.74hij
A13	Rice field		−	0.00g	4.96hi
A14	Rice field	+		2.40f	5.22gh
A15	Rice field	+		2.20f	5.0h
A16	Rice field	+		2.20f	5.28gh
A17	Rice field	+		2.82ef	6.30ef
	In vitro				
A18	Mesophilic stage	+		0.00g	4.20ij
A19	Mesophilic stage	+		2.64f	4.72hij
A20	Thermophilic stage		−	0.00g	4.82hi
A21	Thermophilic stage	+		2.20f	5.24gh
A22	Thermophilic stage	+		3.42de	4.88hi
A23	Maturation stage	+		2.22f	4.50hij
A24	Maturation stage	+		6.18c	15.10c
A25	Maturation stage	+		0.00g	4.60hij

* Values having the same letter(s) in a column do not differ significantly at 5% level of probability

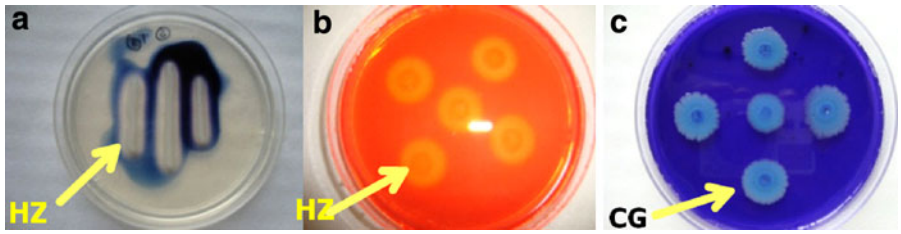

Fig. 1 Ability of actinobacteria (A7) to degrade **a** starch, **b** cellulose and **c** lignin as indicated by the formation of halo zone (HZ) and colony diameter (CG) on media containing starch, carboxymethyl cellulose and azure B

from S-PLUS (Fig. 2). The dendrogram gave three major clusters where overall group similarity ranged from 1.5 (cluster 3) to 8.0 (cluster 1 and 2). Cluster 1 grouped 5 isolates (A2, A4, A7, A9 and A24) with high degree of ability to form halo zones, and well developed colony on media containing starch, CMC and azure B. Cluster 2 consisted of 17 isolates which formed smaller halo zones on starch and CMC and less developed colony on azure B media. Cluster 3 grouped three isolates (A10, A13 and A20) which showed negative response on starch and CMC media, but formed colony on azure B media.

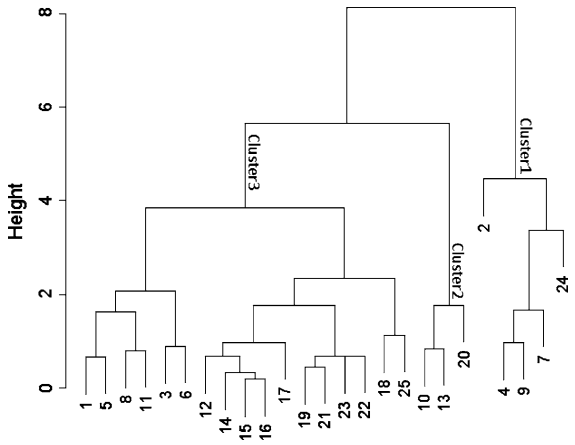

Fig. 2 Dendrogram of 25 actinobacteria isolates based on their lignocellulolytic ability

Lignocellulolytic activity of selected actinobacteria isolates tested on RSP amended media

Five best selected lignocellulolytic actinobacteria (A2, A4, A7, A9 and A24) were evaluated on RSP amended actinobacteria selective media to investigate their adaptability to the substrate. All isolates grew well until 20% level of RSP amendment media, whereas only two isolates (A7 and A9) showed adaptability to 25% level (Fig. 3). Isolate A7 formed the largest clearing zone at 25% level of RSP amended media compared to all other isolates. The higher adaptability to RSP amended media in relation to non-amended media (control) implies that actinobacterium isolate (A7) could efficiently utilize the RSP by producing lignocellulolytic enzymes on such media. Actinobacteria are well known for their ability to decompose complex molecules, particularly lignocellulosic components, which make them important agents in decomposition process. The formation of halo zone on RSP amended media proved that they can produce hydrolytic and oxidative enzymes to break down the complex substrates RSP. These findings were consistent with the results of McCarthy (1987) and Abdulla and El-Shatoury (2007) who applied actinobacteria on rice straw composting and found their efficiency in rice straw decomposition. The highest treatment concentration (25% RSP media) for this study was quite logical because the majority of actinobacteria isolates (A2, A4 & A24) did not cross 20% level except for the isolate A7 and A9. Molla et al. (2002) also used 25% sewage sludge powder media as the highest treatment concentration to screen potential lignocellulolytic fungi. Considering the biochemical characteristics and adaptability to RSP amended cultural media, isolate A7 was identified as *Micromonospora carbonacea* based on Biolog identification system, version 4.20.

Evaluation of isolate (A7) to in vitro composting of rice straw

The decomposition of rice straw resulted in a significant reduction in cellulose, hemicelluloses, lignin and carbon content by *Micromonospora carbonacea* (A7) after 3 weeks of incubation as compared to the control (Fig. 4). After 6 weeks of decomposition, significant difference was found in the amount of cellulose, hemicelluloses and total carbon content between inoculated and control

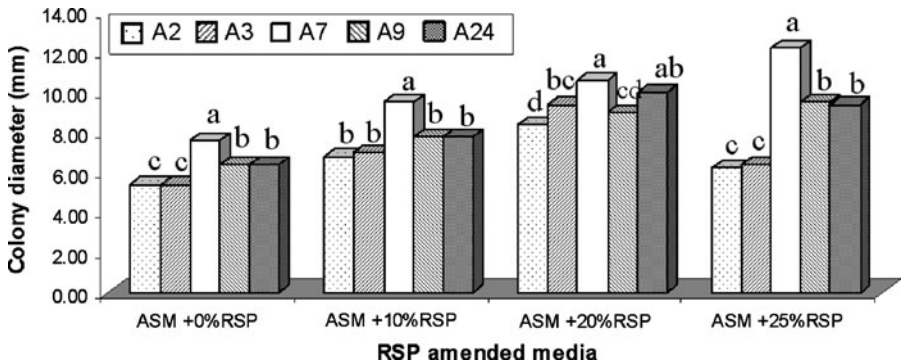

Fig. 3 Growth of five selected lignocellulolytic actinobacteria on rice straw powder amended media. Values having the same letter(s) in a column do not differ significantly at 5% level of probability

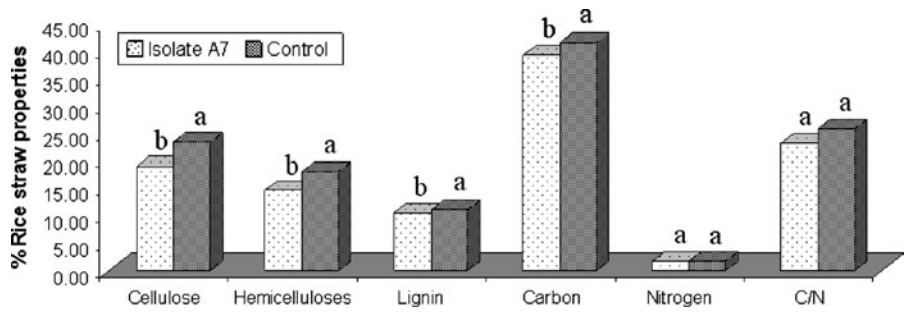

Fig. 4 The content of cellulose, hemicelluloses, lignin, carbon, nitrogen and C/N ratio of rice straw after 3 weeks of composting, values having the same letter(s) in a column do not differ significantly at 5% level of probability

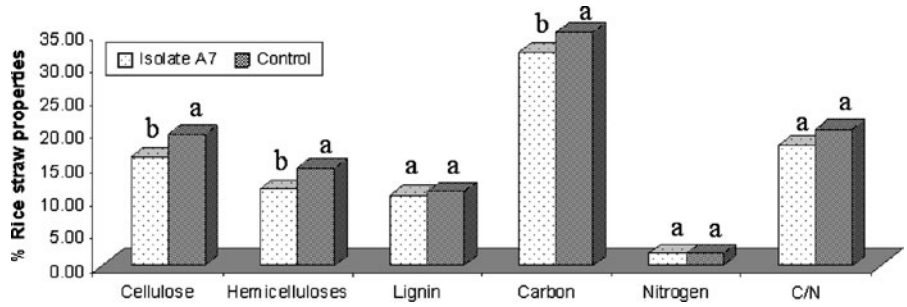

Fig. 5 The content of cellulose, hemicelluloses, lignin, carbon, nitrogen and C/N ratio of rice straw after 6 weeks of composting, values having the same letter(s) in a column do not differ significantly at 5% level of probability

treatments (Fig. 5). The results shown in this study revealed that *M. carbonacea* was able to break down lignin by producing ligninolytic enzymes at the beginning of the composting process. These findings support theoretical expectation and practical experiences that lignocellulolytic actinobacteria produce ligninolytic enzymes at the initial stage of composting process and reach its peak within a few days, making the substrate available for biodegradation (Castillo et al. 1997). The content of cellulose and hemicelluloses in the substrates of inoculated treatment were decreasing and the reducing rate was higher towards the end of the composting process (Fig. 5). These trends of mineralization by lignocellulolytic actinobacterium were expected because after breaking down of lignin barrier cellulose and hemicelluloses were exposed to microbes and they further break down these substrates to glucose producing various hydrolytic enzymes, endoglucanase, exoglucanase, β-glucosidase, xylanase and cellobiohydrolase (Ghose 1987; Tan and Wahab 1997).

During 6 weeks of decomposition, total carbon content decreased in both inoculated and non-inoculated substrates. Microbes utilized carbon compounds as their main energy source. Carbon content is lost during composting in the form of CO_2 as metabolic end-product while total nitrogen content increase due to anabolism of cell structure, enzymes, hormones, etc. The increase of total nitrogen content during composting was in the agreement with other studies (Veeken et al. 2001; Lee et al. 2002) where they showed that straw with sludge amendment increased inorganic nitrogen content to soil. As a consequence, the C/N ratio decreased during the composting period. The C/N ratio is used as maturity index in compost preparation. After 6 weeks C/N ratio of inoculated rice straw compost was 18.1 indicating it was sufficiently mature for field application. *Micromonospora* spp. were well documented, in previous studies, as efficient lignocellulose wastes degrader in aerobic condition (Wenzel et al. 2002; Thawai et al. 2005). Results of the present study also revealed that incorporation of *Micromonospora carbonacea* was an efficient way of composting rice straw.

Conclusions

The results indicate that the lignocellulolytic actinobacteria isolated from natural and induced rice straw compost sources were able to degrade rice straw for rapid composting. Out of 25 isolates, five isolates A2,

A4, A7, A9 and A24 showed the optimum lignocellulolytic potential to starch, cellulose and lignin. Among the five isolates A7 which produced the largest halo zones on 25% rice straw powder amended media was identified as *M. carbonacea,* the best potential lignocellulolytic actinobacterium. In vitro composting study also showed that it could play important role in the large scale composting of rice straw.

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