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Actinomycetes Community from Starch Factory Wastewater

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ABSTRACT

The aim of the research was to study the biodiversity, antimicrobial activity and ability to degrade starch among selected actinomycetes isolates from starch wastewater of rice vermicelli factory. Thirty distinct actinomycetes were identified using their 16S rDNA sequences. Twenty eight strains were classified as *Streptomyces* spp. and two other as *Norcardia* spp. Each of the actinomycetes isolates was tested for their ability to inhibit the growth of other indicator strains, *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC25922 and other two and three phytopathogenic bacteria and fungi. Only one isolate designated WPS132, displayed inhibitory properties against both tested phytopathogenic bacteria and fungi. Phylogenetic analysis showed that this strain was genetically closely related to *S. antibioticus* strain HBUM 174911. The isolates were also tested for their ability to degrade starch. All strains were capable of degrading starch but strain WPS005 showed the highest amylase production at 32.4 U mL^{-1}. We concluded that actinomycetes can be found abundantly from wastewater of the rice vermicelli factory, however, isolates with antimicrobial activities were observed at a low frequency.

Key words: Actinomycetes, starch wastewater, 16S rDNA sequence, biodiversity, phylogenetic analysis

INTRODUCTION

The microbial taxonomic composition in each environmental community is an important indicator of their ecology and function. The biodiversity of bacteria in particular environments can provide insight into what conditions accommodate microorganisms of interest. This information can lead to a better understanding of their function and the environment in which they inhabit (Ghosh *et al*., 2007).

The actinomycetes represent a well-known and extremely diverse group of Gram-positive, filamentous bacteria belonging to the order *Actinomycetales*. Most actinomycetes produce a diverse mixture of hydrolytic enzymes that permit the utilization of various kinds of organic compounds such as starch, cellulose and hemicelluloses. They are recognized as a source of a wide variety of bioactive compounds. Actinomycetes, especially *Streptomyces*, have been most widely studied for the versatility and diversity of useful metabolites they can produce (Berdy, 2005). Thus, most

study on actinomycetes was focused on their potential in producing biological compounds (Kumar *et al*., 2011; Kitouni *et al*., 2005) rather than their function in any specific environment (Bensultana *et al*., 2010; Rintala *et al*., 2002).

Thailand is an agricultural country with many economically important crops such as rice, corn and cassava which are grown and exported. Not only are these agricultural products exported as raw materials but processed products, especially tapioca and rice starches, are produced and exported in large quantities in each year (Sriroth *et al*., 2000). Thus every day, factories processing these agricultural products generate tons of by-products and other wastes. Industrially occurring starch waste is collected in aerobic ponds and prior to the treatment steps required for disposal and may serve as a good source for beneficial bacteria. Generally wastewater discharge from vermicelli factories contain suspended organic pollutants in much concentrations than domestic wastewater and are especially high in starch (Hu, 1989). The accumulation of these compounds in an open-pond system may pose a risk to the health of people living in close proximity to the factory, both due to the nature of the pollutants as well as the potential for the growth of dangerous aquatic organisms. In contrast, these same environments may be capable of supporting several types of bacteria that produce many useful metabolites using high levels of starch as a carbon source (Thavasi *et al*., 2006).

Several groups have isolated actinomycetes producing valuable compounds from varying sources (El-Shirbiny *et al*., 2007). The richness of actinomycetes in an environment appears to reflect the abundance of the resources as well as the waste treatment process. Recently, actinomycetes from a sand bed wastewater filter were screened for the production of antibacterial compounds to investigate their role in removing wastewater-associates pathogens in this environment. Several other actinomycetes have also been reported to effectively control bacterial and fungal pathogens of agriculturally important crops (Prapagdee *et al*., 2008). However, there are only a few reports on the testing of these actinomycetes strains against agricultural pathogens (Rizk *et al*., 2007).

In this study, actinomycetes from the open-pond lagoon wastewater system of a rice vermicelli factory were isolated and identified. In order to estimate the diversity of isolated actinomycetes, an analysis of 16S rDNA sequence which has been proven to be an effective tool for actinomycetes discrimination, was employed (Thampayak *et al*., 2008). The objective of the present study was to analyze their biodiversity, testing their capacity to degrade starch, as well as their inhibitory activity against phytopathogenic microorganisms.

MATERIALS AND METHODS

The study was conducted during October 2009-April 2010 at Faculty of Science, Mahidol University, Bangkok, Thailand.

Actinomycetes strains and culture conditions: Actinomycetes strains in this study were isolated from 4 wastewater samples containing blackish muddy soil. The samples were collected from a single aerobic pond containing a large amount of starch waste. Three procedures were used to obtain variety of actinomycetes strains from the wastewater, filtration of water samples, serial dilution of sediment and heating of sediment followed by serial dilution. (1) Water samples were filtered through a 0.45 μm pore size membrane and the membrane was placed on screening medium and removed after 4 days of incubation (Hirsch and Christensen, 1983), (2) 1 g of sediments and soil sample were diluted with 9 mL of 0.85% NaCl and dilutions of this mixture were then spread

on the screening medium, (3) Sediment samples were heated at 80°C for 60 min prior to being serially diluted and spread on to the screening medium. Screening for actinomycetes was performed using water-proline selective medium (1% proline, 1.2% agar and tap water) and Pridham's agar (1% glucose, 1% glucose, 0.2% $(NH_4)SO_4$, 0.3% $CaCO_3$, 0.1% K_2PHO_4, 0.1% NaCl, 1.2% agar and deionized water). Media were supplemented with cycloheximide (50 μg mL^{-1}) and nalidixic acid (20 μg mL^{-1}) to inhibit the growth of fungi and other bacteria, respectively. Plates were incubated at 30°C for up to one month. Each isolate was purified by streaking on Waksman's agar (Waksman *et al*., 1946) (1% glucose, 0.5% peptone, 0.5% meat extract, 0.3% NaCl, 1.2% agar and deionized water) and were maintained at 30°C for 7 days for colony development and general morphology examination. Strains were maintained on slants of Waksman's agar and Seino agar (1% starch, 0.3% N-Z amine, 0.3% yeast extract, 0.1% meat extract, 1.2% agar and deionized water) and long term storage was in 20% glycerol at -80°C.

Characterization and molecular identification of actinomycetes: The number of actinomycetes species presented in an extremely polluted open-pond wastewater system of a starch factory was investigated, using a plate-culturable method (Zin *et al*., 2010). The actinomycetes isolates were characterized according to their general macromorphology, growth characteristics, colors of aerial spore mass, substrate mycelium and the soluble pigments production (Williams *et al*., 1983) in both Waksman's and Seino agar plates. The 30 distinct actinomycetes isolates were then further characterized by examining the sequences of the 16S rRNA gene.

Chromosomal DNA used as the template for PCR was prepared by a simple boiling method using 16S rRNA gene universal primers UFUL and URUL as forward and reverse primers (Avaniss-Aghajani *et al*., 1996), respectively. The 16S rDNA sequences of actinomycetes isolates were deposited in GenBank under the accession numbers GU581285-GU581314.

Phylogenetic tree analysis: Ribosomal gene sequences from the actinomycetes isolates were compared with those available in Genbank database (http://www.ncbi.nih.gov). Multiple sequence alignments and phylogenetic tree construction was performed by using the MEGA 4 program (Tamura *et al*., 2007). Determination of genetic distances among the sequences was carried out by the Neighbor-Joining method (Saitou and Nei, 1987). Bootstrap analysis with the resembling method was performed as previously described (Felsenstein, 1985) with 1,000 replications.

Detection of antibacterial and antifungal activities: The actinomycetes isolates were also tested for antibacterial and antifungal activities against both standard strains and phytopathogenic strains using co-cultivation method (Bredholdt, *et al*., 2007) as shown in Table 1. Bacterial (*Xanthomonas campestri* and *Erwinia carotovora*) and fungal (*Colletotrichum gloeosporioides* DOA

Table 1: Indicator bacteria and Fungi

Microorganisms	Sources[a]
1. *Staphylococcus aureus* 25923	ATCC
2. *Escherichia coli* 25922	ATCC
3. *Erwinia carotovora* pv. *carotovora*	DOA
4. *Xanthomonas campestris* pv. *campestris*	DOA
5. *Colletotrichum gloeosporioides* c1060	DOA
6. *Colletotrichum gloeosporioides* d0762	DOA
7. *Colletotrichum capsici* c1511	DOA

[a]ATCC: American Type Culture Collection, DOA: Department of Agriculture, Ministry of Agriculture and Co-operatives, Thailand

d0762, *C. gloeosporioides* DOA c1060 and *C. capsici* DOA c1511) pathogens were purchased from Bacterial and Fungal laboratories, Plant Pathology and Microbiology Division, Department of Agriculture, Ministry of Agriculture and Co-operatives. Antifungal activity was evaluated as the distance from the edge of colonies (Bredholdt *et al*., 2007).

Starch degradation and determination of α-amylase activity: The capacity of the actinomycetes isolates to degrade starch was monitored by observing starch hydrolysis (Seibold *et al*., 2006). Isolates producing clear zone were picked and further analyzed for amylase activity in culture broth.

α-Amylase activity in culture supernatants was assayed by measuring the release of reducing sugars (Miller, 1959; Seibold *et al*., 2006).

RESULTS AND DISCUSSION

Morphology and genetic assignment of actinomycetes isolates: Different isolation methods coupled with selective media and culturing conditions were used to segregate actinomycetes species. In such polluted wastewater, 135 actinomycetes isolates were successively recovered from different samples, a large number of which were obtained from slightly basidic conditions (pH 8, data not shown). These results confirm that many actinomycetes can be found in neutral or slightly alkaline environments (Basilio *et al*., 2003; Selyanin *et al*., 2005). In addition, the filamentous in nature of bacteria in this group, increasing the probability of discovering actinomycetes from soil or sediment samples compared with water samples (Bensultana *et al*., 2010). In this study, more numbers of isolates were found in sediment (117/135) than in wastewater (18/135) which coincided with the above report. Based on the present investigation of colony morphology, such as shape, edge and elevation characteristics, as well as colony color, 30 distinct actinomycetes isolates were selected for further characterization by sequencing the 16S rRNA gene. Based on this analysis, 28 isolates were identified as belonging to *Streptomyces* spp. (96-100% similarity) and 2 were *Norcardia* spp. (99.5% similarity) (Table 2). *Streptomyces* spp. were also found predominantly among actinomycetes isolated from starch wastewater in other study (Tapia and Simoes, 2008). Phylogenetic tree relationship of the isolates and other well-characterized actinomycetes species was shown in Fig. 1. The 28 isolates belonging to *Streptomyces* were highly diverse and affiliated into 10 different cluster groups. The major cluster of *Streptomyces* isolates was classified into the following groups, *S. massasporreus* (9 isolates, cluster I), *S. griseoloalbus* (5 isolates, cluster IV), *S. fradiac* (3 isolates, cluster VI), *S. tritolerans* (3 isolates, cluster IX) and *S. thermolineatus* (3 isolates, cluster X), whereas 5 isolates were singly affiliated into clusters (cluster II, III, V, VII and VIII, respectively). The dispersion of isolates in various clusters may reflect the abundance of *Streptomyces* which can be found in any environments (Kitouni *et al*., 2005), including the open-pond wastewater system examined in this study. Thus, the high frequency of *Streptomyces* in the waste environment may indicate that they are well adapted to live in diverse conditions. Of interest to concern regarding potential hazards to human health was the identification of two isolates of *Norcardia* that were closely related to *N. brasiliensis* (Fig. 1) an opportunistic human pathogen known to cause inflammatory disease (Fukuda *et al*., 2008).

Antibacterial and antifungal activities: Seventeen of the actinomycetes isolates failed to show any inhibitory activity against the indicator microorganisms and none of the isolates displayed inhibitory activity against *E. coli*. However, four isolates were capable of inhibiting the growth of

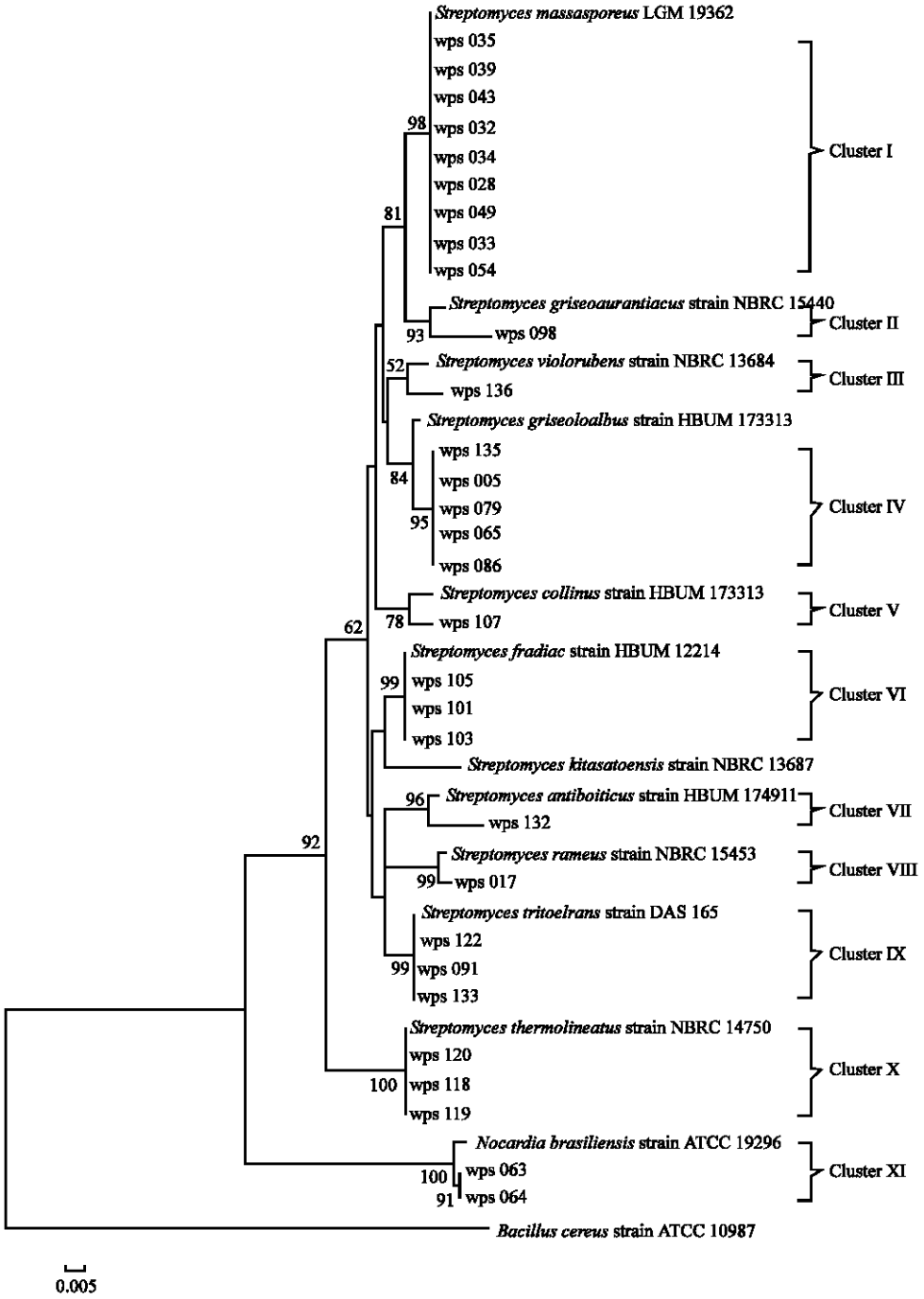

Fig. 1: Neighbor-joining tree based on distance analysis representing relationship between the 16S rDNA sequences of 30 isolates derived from waste reservoir of starch factory. Thirteen sequences of actinomycetes were obtained from Genbank. Bootstrap values generated from 1,000 replicates are shown at the nodes (values over 50% were shown). The sequence from *Bacillus cereus* strain ATCC 10987 was used as the out-group species. Saenna *et al.*

S. aureus and five were inhibitory to both *S. aureus* and fungi (DOA d0762 and DOA c1511). In addition, 7 isolates were active against at least one of indicator fungi (Table 2). Only the WPS

Table 2: Actinomycete isolates and their amylase and antimicrobial activities against indicator microorganisms

Isolate No.	Accession No.	Nearest relatives (% identity)	Amylase activity[1] (U mL^{-1})	Inhibitory against[2] (mm)
WPS005	GU581285	*S. phaeoluteichromatogenes* NRRL 5799 (99)	32.4	a(10), f(9)
WPS017	GU581286	*S. tricolor* LMG 20328 (99.5)	5.3	a(12), f(7)
WPS028	GU581287	*S. massasporeus* NBRC 12796 (99.6)	4.1	a(10), f(8)
WPS032	GU581288	*S. massasporeus* NBRC 12796 (97)	9.9	a(11)
WPS033	GU581289	*S. massasporeus* NBRC 12796 (98)	2.5	-[3]
WPS034	GU581290	*S. massasporeus* NBRC 12796 (99)	5.8	-
WPS035	GU581291	*S. massasporeus* NBRC 12796 (98)	10.8	-
WPS039	GU581292	*S. massasporeus* NBRC 12796 (97)	2.4	-
WPS043	GU581293	*S. hawaiiensis* ISP 5042 (100)	3.7	a(10)
WPS049	GU581294	*S. massasporeus* NBRC 12796 (96)	7.5	-
WPS054	GU581295	*S. massasporeus* NBRC 12796 (99)	9.9	a(9)
WPS063	GU581296	*N. brasiliensis* ATCC 19296 (99.5)	8.6	f(11)
WPS064	GU581297	*N. brasiliensis* ATCC 19296 (99.5)	7.3	-
WPS065	GU581298	*S. purpurascens* SM2-55 (99)	12.1	-
WPS079	GU581299	*S. phaeoluteichromatogenes* NRRL 5799 (98)	22.1	f(10)
WPS086	GU581300	*S. phaeoluteichromatogenes* NRRL 5799 (96)	1.7	-
WPS091	GU581301	*S. tritolerans* DAS 165 (100)	18.2	-
WPS098	GU581302	*S. jietaisiensis* FXJ46 (97.8)	25.5	-
WPS101	GU581303	*S. coelicoflavus* NBRC 15399 (98)	8.5	-
WPS103	GU581304	*S. coelicoflavus* NBRC 15399 (97)	11.0	a(13)
WPS105	GU581305	*S. coelicoflavus* NBRC 15399 (99)	15.9	f(12)
WPS107	GU581306	*S. violaceochromogenes* IFO 13100 (98.6)	6.6	a(10), f(11), g (13)
WPS118	GU581307	*S. thermolineatus* DSM 41451 (97)	18.2	-
WPS119	GU581308	*S. thermolineatus* DSM 41451 (99)	12.7	-
WPS120	GU581309	*S. thermolineatus* DSM 41451 (98)	9.1	-
WPS122	GU581310	*S. tritolerans* DAS 165 (98)	9.1	-
WPS132	GU581311	*S. antibioticus* HBUM174911 (97)	29.4	a(10), c(13), e (18)
WPS133	GU581312	*S. tritolerans* DAS 165 (100)	2.6	-
WPS135	GU581313	*S. phaeoluteichromatogenes* NRRL 5799 (98.6)	1.9	-
WPS136	GU581314	*S. pharetrae* CZA14 (99.7)	1.2	f(10)

[1]Activity was measured in cell free culture broth. [2]a, *S. aureus* ATCC 25923; b, *E. coli* ATCC 25922; c, *X. campestris* pv *campestris*; d, *E.carotovota* pv *carotovora*; e, *C. gloeosporioides* DOA c1060; f, *C. gloeosporioides* DOA d0762; g, *C. gloeosporioides* DOA c1511. The number in parenthesis indicates the zone of inhibition (in mm) including diameter of cork borer (for bacteria a-d) or distance between edges of fungal colony and actinomycetes agar block (in mm) at day 7 (for fungi e-g) (see Materials and Methods). [3]No activity against all indicator microorganisms

132 isolate showed the activity against both indicator phytopathogenic bacteria and fungi but though its inhibitory activity was rather weak. The compound(s) which is involved in the inhibitory activity was not determined but the nature of the antibacterial and antifungal compounds may be distinct in each actinomycetes strain (Bredholdt *et al*., 2007; Egan *et al*., 2001). Interestingly, one of the actinomycetes strain isolated falls into the same cluster as *S. antibioticus* (Fig. 1) which is capable of producing actinomycin (Fawaz and Jones, 1988). Since very few of the actinomycetes isolates displayed any antagonistic activity against indicator microorganisms, it is suggested that these strains have most likely adapted themselves to live in the constrained environment of the open-pond wastewater system where competition with other microorganisms is limited (Bensultana *et al*., 2010). The similar finding of low proportion of actinomycetes or other bacteria

with antimicrobial activity from waste reservoirs were also reported (Schomburg and Muller, 1984; Bal and Dhagat, 2001). Furthermore, all of these isolates showed low activity in starch degradation even though they were isolated from conditions containing high level of starch deposition. The composition of waste and pH had an effect on the activity of amylase (Arotupin, 2007). In *S. rimosus* both α-amylase and glucoamylase activities were lower in submerged culture when compared with solid state cultivation (Yang and Wang, 1999). The low amylase activity of actinomycetes from starch wastewater in this study might be due to both culturing medium composition and culture condition. In addition, bacteria in this group are known to have decreased amylase production in liquid cultures due to fragmentation of mycelium which generally occurs in *Streptomyces* (Simpson and McCoy, 1953).

CONCLUSION

This is the first report in Thailand to show that actinomycetes can be isolated from an open-pond waste system. The majority of the actinomycetes isolated are *Streptomyces* though some are *Nocardia* and the *Streptomyces* isolates show a high level of diversity. However, a few of the isolates displayed antagonistic activity against indicator microorganisms. The results suggest that actinomycetes from starch waste pond have most likely adapted themselves to live in the polluted environment instead of producing compounds to inhibit the growth of other microorganisms. An important aspect of this finding is the identification of *Nocardia* in the factory wastewater. The result of this investigation highlights the need for awareness of the microorganism present to facilitate the waste treatment process and show the importance of increasing the understanding of the relationship of these bacteria in the waste environment.

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