CHAPTER 5

DISCUSSION

5.1 Isolation of effective microorganisms producing potential cellulolytic, amylolytic, proteolytic and lipolytic enzymes

5.1.1 Cellulose-degrading microorganisms

In our experiment we isolated 20 strains which produced large clear zones on the carboxy methyl cellulose agar (CMC) due to the degradation of the carboxy methyl cellulose. The cellulase productivities of the isolates i.e. 10 bacteria, 5 actinomycetes and 5 fungi with clear zone ranging from 15 - 22 mm were tested for enzyme activity. We selected 4 strains i.e. LHE 3, LHE 10, LHE 12 and LPA 15 showing strong activities. They grew well at high temperatures and produced more than 0.05 U ml^{-1} of cellulase in the culture filtrate. Strain LHE 3, LHE 10, LHE 12 and LPA 15 appeared to belong to the species, *Bacillus subtilis*, unknown fungus, *Aspergillus flavus* and *Streptomyces regensis*, respectively.

It is known that cellulase system contains endoglucanase (1,4-β-glucan glucanohydrolase, EC 3.2.1.4), exoglucanase (1,4-β-glucan cellobiohydrolase) and β-glucosidase (β-D-glucoside glucohydrolase or cellobiase). Exoglucanase is necessary for splitting off the elementary fibrils from the crystalline cellulose (Fan and Lee, 1983; Schewale, 1982; Woodward and Wiseman, 1983), but only the synergy of the above enzymes makes possible the cellulose hydrolysis to glucose (Ryu and Mandels, 1980; Sandhu and Bawa, 1992; Wood, 1989; Wood and McRae, 1978; 1979) or a thorough mineralization to H_2O and CO_2. Many fungi are able to break down polysaccharides such as celluloses and convert these polymeric compounds into sugars due to their capability to produce extracellular enzymes, and cellulase research was mainly focused on fungi in the past (Akin, 1987; Mandels, 1982; Petre *et al*., 1999; Rosevear, 1984; Wood, 1992). Few bacteria possess a complete multi-enzyme system for lignocellulose degradation; however, there has been increasing interest in cellulase production by bacteria because of the fast growth rate and many cellulolytic bacteria were isolated from various environments (Coughlan, 1989; Li and Gao, 1997; Mandels and Reese, 1985; Petre *et al*., 1999). As previous studies, the results had showed enhanced lignocellulose biodegradation during the composting of flower stalk and vegetable waste when inoculated with the primary mixed culture (Huang *et al*., 2004; Lu *et al*., 2004), the result in this study indicated that enhanced cellulose degrading ability was due to the complementarity of cellulases from different strains.

This present study showed that *Bacillus subtilis* LHE 3, unknown fungus LHE 10, *Aspergillus flavus* LHE 12 and *Streptomyces regensis* LPA 15 had higher CMCase activity. The genus *Bacillus* consists of a group of aerobic or facultatively anaerobic bacteria with a wide diversity of physiological ability with respect to heat, pH and salinity. Many species are normally present in soil and in decaying animal and vegetable matter (Holt, 1994; Daniel and Nilsson, 1998). Other studies demonstrated that *Bacillus* species played important roles in biodegradation and bioconversion of big molecular compounds (Akin, 1987; Holt, 1994; Rosevear, 1984), and *Bacillus subtilis* with 0.105 U mL^{-1} cellulase activity as well as *Bacillus* *licheniformis* with 0.118 U mL^{-1} cellulase activity are frequently reported for cellulolytic species (Liu *et al*., 2004; Petre *et al*., 1999). The important cellulolytic fungus like *Trichoderma* sp. (Wood and McCare, 1972; Shaw and Quejesky, 1979; Mandels and Reese, 1985), *Penicillium* sp. (Hoffman and Wood, 1985; Brown *et al.*, 1987), *Sporotrichium* sp. (Erikkson and Johnsrud, 1983), *Aspergillus* sp.(Bennet, 1998. Kazuhisa, 1997) etc. have been reported to have cellulolytic activity. Some actinomycetes such as species of *Streptomyces* were found to be active cellulose decomposers and therefore useful for waste disposal which often found during composting process (Crawford and McCoy, 1973; Stutzenberger, 1971). The isolation of *Bacillus subtilis*, unknown fungus, *Aspergillus flavus* and *Streptomyces regensis* of this research is probably another evidence for adaptation and used the mixed culture of cellulolytic microorganisms in large-scale composting inoculants.

5.1.2 Starch-degrading microorganisms

We isolated 12 strains which produced large iodine clear zones between 25 - 35 mm on the starch agar plates due to the degradation of carbohydrate in soluble starch. The amylase productivities of the 12 isolated bacteria were tested in the liquid medium for 24 hours. After the cells have been removed by centrifugation (10000g, 15 min), the culture solution was taken off and used as the crude enzyme preparation. All tested isolates produce amylase and their activities ranged from 0.0606 - 0.2559 × 10^{-3} U mL^{-1}. We selected only 1 strain GB 12 showing strong activities for use as compost inoculum. They grew well at relatively high temperatures (50°C) and produced 0.2559 × 10^{-3} U mL^{-1} of amylase in the culture filtrate. This isolate was identified by morphological and biochemical characteristics and DNA sequencing as *Bacillus* strains, resembling *Bacillus subtilis*.

Many microbial enzyme preparations have been investigated on the starch digesting activity, however, only limited attempts to search for microorganisms which produce starch digesting enzyme have been reported. Taniguchi *et al*. (1982) and Ishigami *et al*. (1985) found *Bacillus circulans* F-2 strain from potato starch and *Chalara paradoxa* from pitch of sago palm, respectively. In this study, we attempted to isolate microorganisms with high activity for starch by using a soluble starch as a sole carbon source, for the isolation medium. In conclusion, the enzyme preparation of the strain GB 12, selected in this study, is high effective to hydrolyse starch. Thus, it is the most suitable for application in preparation of compost inoculum.

5.1.3 Protein-degrading microorganisms

In this present study we isolated 10 strains which produced large clear zones on skim milk agar due to the degradation of the nitrogenous compound in skim milk. The protease productivities of bacteria isolates were tested for enzyme activity. We selected just one strain of isolate BS 1 showing strong activities. They grew well at high temperatures and produced 0.2508 U mL^{-1} of protease in the culture filtrate. This isolate was identified by morphological and biochemical characteristics and DNA sequencing as appeared to belong to the species *Bacillus subtilis*. This value is much higher than those of *Bacillus subtilis* (0.0526 U mL^{-1}), *B. stearothermophilus* (0.1879 U mL^{-1}) and *Thermus aquaticus* (0.0885 U mL^{-1}) reported elsewhere (Mauriello and Villani, 2002). We believe that the protease produced by our isolate is comparable to or even better than the protease from *B. stearothermophilus* (0.1074 U mL^{-1}). Our strain would make its eligible as a good starter cultures for composting process.

5.1.4 Lipase-degrading microorganisms

In this present study we isolated 11 strains which produced large clear zones on tributyrin agar due to the degradation of the tributirin. The lipase productivities of bacteria isolates were tested for enzyme activity. We selected only 1 strain of isolate LPC 2 showing strong activities. They grew well at high temperatures and produced 97.765 U mL^{-1} of protease in the culture filtrate. This isolate was identified by morphological and biochemical characteristics and DNA sequencing as appeared to belong to the species *Bacillus subtilis*. This enzyme levels are comparable to or higher than those of some common mesophilic lipase-producing bacteria (Ghanem *et al*., 2000).

The practice of producing organic fertilizer through the biological decomposition of organic wastes has been carried on for centuries as an art generally known as composting. Mostly the substrates of composting with primary components of plant material such as cellulose, hemicellulose and lignin are rather difficult to biodegrade and reduce the availability of the other polymers by means of a physical restriction (Ladisch *et al.*, 1983). Actually, it caused a problem of long-termed processing for decomposition. Then many researchers have selected and applied cellulolytic microorganisms as an inoculum of composting to speed up the natural process to obtain a better final compost eventhough it has been a controversial subject for a long time. In fact, contradictory results have been described by different authors (Golueke *et al.*, 1954; Kosinkiewiez, 1974; Finstein and Morris, 1975; Wani and Shinde, 1978; Solbraa, 1984; Nakasaki and Akiyama, 1988; Magan *et al*., 1989; Kostov *et al*., 1991; Requena *et al*., 1996; Rajbanshi *et al*., 1998; Elorrieta *et al*., 2002; Nakasaki *et al*., 2005). Nevertheless, plant wastes are not only composed of cellulose but also other components of starch, protein and lipid (Alexander, 1961). Generally, these components will be degraded by natural microorganisms in organic wastes easier than cellulose. However, the addition of effective organisms for such substrates can prepare much more nutrients for abundant microflora to establish proper composting conditions so that the required composting process starts and precede ealier (Tam and Vrijmoed, 1990; Fukuda, 1991; Chan *et al*., 1994).

5.2 Compost inoculum development

5.2.1 Proper concentration of effective microorganisms for decomposing vegetative matter

Three compost inocula i.e. Formula 1 (10^5 CFU g^{-1}), Formula 2 (10^6 CFU g^{-1}) and Formula 3 (10^7 CFU g^{-1}) belonging to different concentration of the same microorganisms and Market brand inoculum were analysed for their ability of decomposing steriled rice straw (as model of agriculture wastes) and one of them would used as proper inoculant for actual composting. During the decomposition processes, total counts, C/N ratio and pH were determined as a matter of course. The growth of thermophilic bacteria, fungi, actinomycetes and cellulose-degrading microorganisms were studied during decomposition of rice straw. In all treatments, as microbial activities proceeded, the growth of related microorganisms declined after reaching a peak due to depletion of the accessible energy sources. (Ten *et al*., 1988). In the present study, the microbial populations of treatment with Formula 3 inoculum was much more than those of other treatments during the whole decomposting period. Probably, it was dependent upon a larger inoculum size in Formula 3. This result also happened to the research of Shin *et al.*(1999), who reported that inoculation of garbage using specialized microorganisms increased microbial activity. As for Formula 2 inoculum, the concentration of microorganisms was equal to Market brand inoculum. The growth of thermophilic bacteria, cellulose-degrading microorganisms and actinomycetes of both treatments conducted in the same trend all over the decomposition period but the treatment with Formula 2 gave the growth of thermophilic fungi higher than that with Market brand inoculum. This result can be determined by the C/N ratio of treatment with Formula 2 inoculum which significantly decreased faster than the latter one. In addition, effective microorganisms from Formula 2 inoculum could increasingly grow in decomposting rice straw.

5.3 Composting process

5.3.1 Decomposing rice straw in laboratory experiment

A succession of microbial populations was observed during the decomposing process. The growth of total thermophilic bacteria, fungi, actinomycetes and cellulose-degrading microorganisms of decomposing with addition of inocula (Market brand and CMU) significantly increased in number more than decomposing without inoculum all over the time of decomposing process. As reported by Beidou *et al.* (2005), he stated that inoculation would be an effective way to improve the microbial concentration and in turn increase the composting rate. From our results, the growth of total thermophilic fungi and actinomycetes growth in decomposing with Market brand inoculum was rather lower than that in decomposing with CMU inoculum but not significantly different. In addition, the growth of thermophilic bacteria and cellulose-degrading microorganisms were similar for both decomposting with Market brand and CMU inocula. It noted that, although the changes of some microbial growth in decomposing with CMU inoculum was similar to decomposing with Market brand inoculum but the C/N ratio of decomposting with CMU inoculum significantly accomplished maturity of decomposition of rice straw prior to that decomposting with Market brand inoculum. This may be because of CMU inoculum contained an effective microorganisms, which could quickly decompose soluble and biodegradable organic matter such as mono-saccharides, starch, lipids and proteins (Tiquia *et al.*, 1997). The change of C/N ratio is one of parameters, which is frequently used as index of maturity. When a waste is decomposed, generally there is decrease in C/N ratio with time due to losses of C as CO_2 which stabilizes in the range of 15-35 (Poincelot, 1974; Golueke, 1981). In the present studies, the C/N ratios after 60 days of composting with CMU inoculum was in this range. Likewise pH change from alkaline to neutral level in decomposing treatment with CMU inoculum took precedence over the others. It implied that decomposition with CMU inoculum succeeded in maturity prior to the others (Oliveira *et al.*, 2002).

5.3.2 Composting in field experiment

Nowadays, many compost inoculum products have been developed to enhance the fermentation process within the composting process (Lin, 1991; van Schaijk, 1991; Chaw, 1996). However, the microorganisms inocula and their role in composting have been controversial issues over the years (Nakasaki and Akiyama, 1988). From the results of Nakasaki and Akiyama (1988), this investigation revealed that addition of compost inoculum in the composting system enhanced the rate of composting of the sawdust in windrow. In this trial, there were very similar trends of physical, chemical, and microbiological factors during composting between Market brand and CMU inocula and these were significantly different from control piles (without compost inoculum) all over the composting time. The growth rates of thermophiles and mesophiles of bacteria, fungi, actinomycetes and cellulose-degrading microorganisms in compost piles with Market brand and CMU inocula were significantly($p<0.05$) faster than those of control piles did. Probably, the addition of effective organisms from inocula was active and prompted to accelerate processing of decomposing organic matter in composting piles. Golueke *et al*. (1954) reported that the addition of compost inoculum is of value in composting only if the bacterial population in the piles is unable to develop rapidly enough to take full advantage of the compost pile's capacity to support bacterial growth. In addition, microbial succession plays a key role in composting process and appearance of some microorganisms reflects the quality of maturing compost (Ishii *et al.*, 2000; Ryckeboer and Mergaert, 2003). On the other hand, the development of mesophilic and thermophilic bacteria, fungi, actinomycetes and cellulose-degrading microorganisms during composting are related to the mesophilic and thermophilic stages of the composting system (Diaz-Ravina *et al*., 1989; Davis *et al*., 1991; Ishii *et al*., 2000; Riddech *et al*., 2002).

Temperature has long been recognized as a key environmental factor affecting microbiological activity. In general, each group of microorganisms has an optimum temperature and any deviation from it results in a fall in activity and growth (Golueke, 1972). In composting, mesophilic microorganisms use readily available carbohydrates and decomposable proteins (Poincelot and Day, 1973). Heat is generated by heterotrophic bacteria (Baines *et al*., 1985). The temperatures throughout the piles with Market brand and CMU inocula rose very rapidly from ambient to 60-65°C within 2 days. Higher increase in temperature have been shown with North American hardwood barks (Hoitink *et al*., 1978); imported barks in Japan took less than 10 days to reach maximum temperatures (Hong and Ueyama, 1973); the maximum peak occurred within 4-5 days in straw composts (Chang and Hudson, 1967). The temperature gradient decreased from the centre of the piles to the perimeter, which is to be expected since the heat in the centre of the pile is well insulated. Heat loss and generation are proportional to the surface area of the pile. Therefore, the larger a pile, the smaller the surface-area-to-volume ratio and the less the heat loss (Golueke, 1972). The initially rapid rise in temperature of compost piles with Market brand and CMU inocula were relatively short-lived and after 60 days of composting, temperatures of compost piles with CMU inoculum had fallen to ambient temperature(29°C) significantly faster than compost piles with Market brand inoculum did. The fall in temperature might be due to lack of readily available substrate for the microorganisms to use. Temperature has been shown to be a critical determinant of composting efficiency (Finstein *et al*., 1986). It has been reported that the increase in temperature during the early stage of composting may be as the result of thermophilic phase of composting (Ros *et al*., 2006). The temperature during composting could rise as a consequence of the rapid breakdown of the readily available organic matter and nitrogenous compounds by microorganisms.

The pH of the all piles gradually decreased may be due to a lot of organic carbon content in these piles. The reduction in pH in the compost piles might be due to the fact that organic carbon was degraded to organic acid by the acid-forming bacteria existing in the compost pile (FAO, 1987). In addition, the pH decrease might be caused by the mineralization of organic acid during the composting process as well as the large quantities of carbon dioxide released during the composting process (FAO, 1987; Tiquia *et al*., 1996; Tiquia *et al*., 1997; Tiquia *et al*., 1998; Huang, 2004; Meunchang *et al*., 2005). Tiquia, (2002) studied the changes in composting of spent pig manure and sawdust litter. They reported that the change of composting temperature had a strong correlation with some chemical parameters such as the pH and decided to use these parameters to determine the maturity of compost. Our results of pH change in compost piles with CMU inoculum rapidly entered to pH level of 7 prior to the other piles (control piles and compost piles with Market brand inoculum). At the pH level around 7, it indicated that composting pile become a completely compost (Ouatmane *et al*., 2000).

Moisture content is an important parameter influencing biological activity and biochemical rates. The initial percentage of moisture content of all piles were adjusted at 60 % and water was added every week to conserve moisture content at 60% . It was reported that suitable and efficient moisture content in composting of spent litter was between 50 % and 60 % because an optimum level of moisture content had strong effect on oxygen consumption rate of aerobic heterotroph microorganisms (Tiquia *et al*., 1996). In our experiment the moisture content dropped gradually during composting time. A decrease in moisture content was due to the release of moisture from the compost pile through water evaporation as a result of the heat generated from microbial activities during composting (Miller and Finstein, 1985; Cunha *et al*., 2002). When the temperature in the compost piles reached the ambient temperature, addition of water was stopped although the composting process still continued. With respect to moisture content, the compost piles with CMU inoculum after 60 days of composting period have early achieved an acceptable level of quality of mature compost with less than 50% moisture content (Ta-oun *et al*., 2005).

The initial C/N ratio was the main factor affecting the time of the compost maturity (Tiquia *et al*., 1997). The ideal carbon-to-nitrogen (C/N) ratio for composting is generally considered to be around 30:1. C/N ratios lower than 30:1 allow rapid microbial growth and speedy decomposition, but excess nitrogen will be lost as ammonia gas, causing undesirable odors as well as loss of the nutrient. C/N ratios higher than 30:1 do not provide sufficient nitrogen for optimal growth of the microbial populations. This causes the compost to remain relatively cool and to degrade slowly, at a rate determined by the availability of nitrogen (Schnitzer *et al*., 2000). However, the C/N ratio is also one of the factors used for indicating compost maturation. Composts with the C/N ratio of not more than 20 are required of their maturation (Land Development Department, 2005). Reports about the C/N ratio of some compost at a maturity phase stated 13.27 for the co-composting of chestnut burr and leaves with solid poultry manure (Guerra-Rodriguez *et al*., 2001) and 11-17 for the composting of bagasse with sewage sludge (Negro *et al*., 1999). With regard to the C/N ration in this experiment, the maturity of compost expressed at low level of about 13-20 then, proper time for composting maturity had to calibrated by other parameters, escially at equilibrium temperature of inside and outside composting pile.

Various hydrolytic enzymes are believed to control the rate at which various substrate are degraded. Enzymes are the main mediators of various degradative processes (Mckinley *et al*., 1985; Tiquia *et al*., 1996; Tiquia, 2002). So the changes in activities of cellulase which is responsible for hydrolysis of cellulose was studied to understand the degradation of organic wastes. . In general, cellulose decomposition limits the rapid production of compost more than any other substrates (Poincelot, 1977). Cellulase activity involved in the degradation of cellulose is dependent on the types of cellulolytic microorganisms that develop on the organic waste (Goyal *et al*., 2005). Mostly, fungi are involved in the decomposition of cellulose, hemicellulose and lignin present in the organic matter. Among the composting treatment, the cellulase activity was found to be higher in compost piles with CMU inoculum. This could have attributed to the initial addition of inoculum which it has effectively cellulose-degrading microorganisms(bacteria, fungi and actinomycetes). This inoculum led to stimulate active decomposition of composting from sawdust. One more reason, which can be suggested for the increase, may be the reduction in C/N ratio in the later stages of normal composting, which allowed greater nitrogen availability (Ashbolt and Line, 1982) and favoring the growth of microbial biomass.

At the end (80 days) of composting process, all measuring parameters i.e. total organic carbon, total nitrogen, total phosphorus, total potassium, total calcium, total magnesium, electrical conductivity (EC) values and germination index of compost treatments with Market brand, CMU inoculum and control approached to the regulation of complete compost and were in standard of Land Development Department, Thailand. Certainly, they could be acceptable and used as fertilizer