CHAPTER 4
DISCUSSION AND CONCLUSION

4.1 Discussion

4.1.1 Screening and characterization of a myrosinase producing fungi

The screening of glucosinolate degrading microorganisms was done using plate method. *Rhizopus* sp., *Mucor* sp., *Aspergillus* sp. and gram negative rod bacteria were isolated from soil samples. In the screening, sinigrin was the main carbon source avalible in the agar medium. Strains capable of growing in this selective medium were noted as sinigrin degrading strains. *Brassica* crops and other glucosinolate containing materials were stated as sources of glucosinolate degrading microorganisms, such as *Aspergillus clavatus* II-9, *Enterobactor cloaceae* no.406 and *Fusarium oxysporum* @146 (Tani *et al.*, 1974; Smith *et al.*, 1993). *Bacillus* sp., *Streptomyces* sp. and *Staphylococcus* sp. have been screened from petrochemical contaminated soil samples by the same method which presented glucosinolate degradation (Brabban and Edwards, 1995).

Regarding the three fungal isolates, the *Aspergillus* sp. showed a potential for degrading sinigrin in liquid culture, while, sinigrin degrading capablilties of the *Rhizopus* sp. and *Mucor* sp. were relatively low. According to previous reports, glucosinolate degrading fungi were *Aspergillus*, including *A. syndowi* IFO 4284 (Ohtsuru *et al.*, 1969), *A. syndowi* QM31c (Petroski and Kwolek, 1985), *A. niger* AKU3302 (Ohtsuru and Hata, 1973), *A. clavatus* II-9 (Smith *et al.*, 1993) and

Aspergillus sp. NR4201 (Sakorn *et al.*, 1999). However, *Fusarium oxysporum* @146 was the only non-*Aspergillus* previously established as a glucosinolate degrading strain (Smith *et al.*, 1993). The 23 sinigrin degrading fungi in screening results here were isolated as *Aspergillus* sp. 21 strains, *Rhizopus* sp. 1 strain and *Mucor* sp. 1 strain. The information on physical morphology, growth characteristics confirmed that *Aspergillus* sp. NR463 was a member of the *flavus* group. We also have these lab results confirmed by MIRCEN (UNESCO World Network of Microbiological Resources Centre), Bangkok Thailand. Fungi of the *flavus* group are comprised with several species such as *A. flavus*, *A. paraciticus* and *A. oryzae*.

In addition to the preliminary assessment of sinigrin degradation in liquid culture, the presence of myrosinase activity in fungal mycelium of the *Aspergillus* indicated the role of this enzyme in the sinigrin degrading process. Whereas, the degradation of sinigrin by *Rhizopus* and *Mucor* did not involve myrosinase since myrosinase activity was not detected in culture media and cell free extracts. These suggestions were supported by the evidence in sinigrin barium agar plates. The absence of fungi on sinigrin barium agar plates presented no opaque zone. Opaque zones are formed surrounding the colonies of the *Aspergillus* in sinigrin barium agar plates (5mM sinigrin, 5 mM ammonium chloride, 2.5 mM barium chloride and 1.5 g per lit agar in deionized water) (Sakorn *et al.*, 2002). The formation of this opaque zone indicated the action of myrosinase that released sulfate from sinigrin into the

agar medium, which then formed the insoluble barium sulfate salt. On the other hand, such formations were not presented by *Rhizopus* sp. and *Mucor* sp. The degradation of sinigrin by *Rhizopus* sp. and *Mucor* sp. resembled that of *F. oxysporum* @146, which was also not associated with myrosinase (Smith *et al.*, 1993).

Myrosinase activity of the *Aspergillus* was produced exclusively when the strain was cultured in the medium containing glucosinolate (sinigrin). This indicated that an inducible feature of this enzyme was present and therefore it resembled other myrosinase producing fungi such as *A. syndowi* IFO4284 (Ohtsuru *et al.*, 1969) and *A. syndowi* QM31c (Petroski and Kwolek, 1985), *A. niger* AKU3302(Ohtsuru *et al.*, 1973), *A. clavatus* II-9 (Smith *et al.*, 1993) and *Aspergillus* sp. NR4201 (Sakorn *et al.*, 1999). At low sinigrin concentration (0.5-2.5 mM), enzyme activity was expressed proportionally. However, expression of the enzyme was not inducible at high concentration levels of sinigrin (2.5-5.0 mM).

There were some interesting points on sinigrin glucose medium of the *Aspergillus*, in comparison to other microbial strains. Firstly, most of the microorganisms (gram negative bacteria, *Rhizopis* sp. and *Mucor* sp.) exhibited high consumption rates for glucose (36 h), whereas, those for sinigrin were very low. The sinigrin consumption was likely due to non-induced cells of *Lactobacillus agilis* R16 (Palop et al., 1995). Secondly, high consumption rates for sinigrin and glucose of *Aspergillus* sp. were exhibited. The results were agreed with other myrosinase

production microorganism which is the characteristic of *Aspergillus* sp. NR4201 (Sakorn *et al.,* 1999). However, these were quite different from *Lactobacillus agilis* R16 (Palop *et al.*, 1995), *Aspergillus clavatus* II-9 (Smith *et al.*, 1993) and *Fusarium oxysporum* @146 (Smith *et al.*, 1993). Induced cells of the *Lactobacillus* and *Fusarium* exhausted glucose before sinigrin, where as, the *A. clavatus* consumed sinigrin prior to consuming glucose. Finally, the occurrence of myrosinase activity in cell-free extracts of the *Aspergillus* resembled those of *A. clavatus* (Smith *et al.*, 1993), *Aspergillus niger* AKU3302 (Ohtsuru *et al.*, 1973), *Enterobacter cloacae* no.406 (Tani *et al.*, 1974) and *Paracolobactrum aerogenoides* (Oginsky *et al.,* 1965). This implied that the in vivo glucosinolate degradation of these microbes was an intracellular process. This study demonstrated that the availability of myrosinase activity in fungal mycelium of the *Aspergillus* was closely related to its degradative potential for the glucosinolate (sinigrin). Opposingly, enzyme activity was not detected in cell free extracts or cultured filtrates of the Lactobacillus (Palop *et al.,* 1995) and *Fusarium* (Smith *et al.,* 1993). Sinigrin degradation by intact cells of *Aspergillus* led to the production of allylcyanide, which resembled that of *A. clavatus* (Smith *et al.,* 1993). By contrast, allylisothiocyanate was produced by the *Lactobacillus* (Palop *et al.,* 1995), whereas, none was produced by the *Fusarium* (Smith *et al.,* 1993). The allylcyanide was not detected in cell free extracts of the *Aspergillus* (data not shown).

During the incubation of whole cells of the *Aspergillus* in mustard extract medium, it was interesting to note that glucose was produced in culture filtrates. In such case, endogenous glucose should not change during the glucose production period. The production of allylcyanide by fungal cells was not detected; however, myrosinase production was related to sinigrin degradation. This indicated that sinigrin was a main, or probably the only glucosinolate contained in brown mustard seeds. Sinigrin served as a sole preferred carbon source for the fungus. A success of the cultivation of the *Aspergillus* in mustard extract medium provided information for enzyme production at lab scale level.

4.1.2 Enzyme production in liquid culture

It is noteworthy that myrosinase was produced from the *Aspergillus* in mustard extract medium with the highest glucosinolate concentration (10mM) ever reported here. Other reports on myrosinase production by such microorganisms as *A. syndowi* (Ohtsuru *et al.,* 1969), *A. niger* (Ohtsuru and Hata, 1973), *E. cloaceae* (Tani *et al.*, 1974) and *Aspergillus* sp. NR4201 (Sakorn *et al.*, 1999) were traditionally employed by low glucosinolate cultures (0.2-5.5 mM). In such cases, materials containing glucosinolates such as mustard seed meals were later introduced into the induction medium (Petroski and Kwolek, 1985; Ohtsuru and Hata, 1973; Ohtsuru *et al.*, 1969; Ohtsuru *et al.,* 1973; Sakorn *et al.,* 1999). However, such induction media did not work well in other cases e.g. the bacterium *E. cloaceae* no. 406, in which, pure

sinigrin was added to a final concentration of 0.02% (Tani *et al.,* 1974). In our cultivation, mustard extract medium was derived from brown mustard cake (*Brassica juncea*) without any nutrient supplementation. Brassica oil seeds were valuable protein sources (Masheshwari *et al.*, 1981; Miller *et al.*, 1962). This was supported by evidence in our laboratory that mustard seeds contained significant amounts of starch and polysaccharides (unpublished results). These polysaccharides probably served as substrates for some exoglucanase enzymes that released glucose into culture filtrates.

Cultured conditions between this one-step culture and the other two-step cultures of *A. syndowi* IFO4284 (Ohtsuru *et al.*, 1969), *A. syndowi* QM31c (Petroski and Kwolek, 1985), *A. niger* AKU3302 (Ohtsuru *et al.*, 1973) and *Aspergillus* sp. NR-4201 (Sakorn *et al.*, 1999) are summarized in table 4.1. One of several advantages of the one step culture was a shorter cultivation period. The cultivation was completed within 48 hr. The two step culture of *A. syndowi* IFO4284 required 17 days, including 3 days for preculture and 14 days for enzyme production (Ohtsuru *et al.*, 1969). In the case of *A. syndowi* QM31c, the culture was employed for 6 days, 3 days for pre-culture and another 3 days for enzyme production (Petroski and Kwolek, 1985). Cultivation for myrosinse production by *A. niger* AKU3302 required 4 days. This included 2 days for preculture and another 2 days for enzyme production (Ohtsuru and Hata, 1973).

The one step cultivation of the *Aspergillus* sp. NR463 showed, myrosinase activity of 68.45 units per 50 ml mustard extract medium (1.369 units per ml of the culture medium). The production of *Aspergillus* sp. NR-4201 was shown, enzyme activity of 35 units was produced in 40 ml mustard extract media (Sakorn *et al.*, 1999). While those two step cultures of the *A. syndowi* IFO4284 (Ohtsuru *et al.*, 1969) and *A. syndowi* QM31c (Petroski and Kwolek, 1985) yielded the activity of 400 units per 5 l medium and 4 units per 50 ml medium, respectively. In case of *A. niger*, enzyme activity of only 4 units was obtained for 100 ml medium (Ohtsuru and Hata, 1973)

Table 4.1 Comparison of myrosinase production by *Aspergillus* sp. NR463, *Aspergillus* sp. NR-4201 (Sakorn *et al.*, 1999), *A. syndowi* IFO4284 (Ohtsuru *et al.*, 1969), *A. syndowi* QM31c (Petroski and Kwolek, 1985) and *A. niger* AKU3302 (Ohtsuru and Hata, 1973).

Item	*Aspergillus* sp.				
	NR463	**NR-4201**	**IFO4284**	**QM31c**	**AKU3302**
Optimal temperature (°C)	30	30	29	25	29
Optimal pH	6.5	6.5	6.5	6.3	7
Cultivation time (days)	1.5	2	17	6	4
Cofactor supplement	None	none	none	none	ascorbic acid
Inorganic salt supplement	None	none	$MgSO_4$	$MnSO_4$, $MgSO_4$ $FeSO_4$, $ZnSO_4$ $CaCl_2$, $CoCl_2$	$CoCl_2$
Enzyme Production (Unit/ml culture medium)	68.45 U/50 ml 1.369 U/ml	35 U/40 ml 0.875 U/ml	400 U/ 5 lit 0.08 U/ml	32 U/330 ml 0.097 U/ml	4 U/100 ml 0.04 U/ml

Ohtsuru *et al.* (1973) reported that supplementation with 0.1% L-ascorbic acid in cultured medium, myrosinase production was significantly increased by *A. niger*

AKU3302. Significant elevation of the enzyme was shown by replacing 0.2% sucrose with 0.1% glucose (Ohtsuru and Hata, 1973). Cobalt chloride was stated as an essential cofactor for myrosinase production by *A. niger* AKU3302 (Ohtsuru and Hata, 1973). Whereas, magnesium sulfate at the final concentration of 0.03% was supplemented for the myrosinase production by *A. syndowi* IFO4284 (Ohtsuru *et al.*, 1969). Magnesium sulfate, manganese sulfate, zinc sulfate, ferrous sulfate, calcium and cobalt chloride were usually used as cofactor for *A. syndowi* OM31c (Petroski and Kwolek, 1985). In the case of *Aspergillus* sp. NR463 and *Aspergillus* sp. NR4201, no metal ions were used.

The crude enzyme of the *Aspergillus* sp. NR463 was quite stable at 4°C. The *Aspergillus* sp. NR463 enzyme exhibited considerable stability, compared to other microbial myrosinase from *Aspergillus* sp. NR4201 (Sakorn *et al.*, 1999), *A. niger* (Ohtsuru *et al.*, 1973) and *E. cloaceae* no.406 (Tani *et al.*, 1974). In the case of *A. niger* AKU3302 enzyme, 1 mM L-ascorbic acid and 10 mM mercaptoethanol supplements could preserve 80% of enzyme activity for 2 days at 20°C (Ohtsuru *et al.*, 1973). Increased enzyme stability of *Aspergillus* sp. NR4201 was shown with the presence of 1 mM mercaptoethanol (Sakorn *et al.*, 1999).

4.1.3 UV mutagenesis of *Aspergillus* sp. NR463

The survivors of UV mutagenesis of wild-type *Aspergillus* sp. NR463, were selected for myrosinase hyperproduction by observing the opaque zone on sinigrin-barium agar plates, growth profiles on mustard extracted media and activity and stability of the enzyme. The results of the mutagenesis gave 5 candidate mutant strains. The mutant strains NR463U1, NR463U2, NR463U3, NR463U4 and NR463U5 had activities of 1.10, 1.85, 1.70, 2.35 and 1.15 units ml^{-1} respectively, which had 81.48, 137.04, 125.93, 174.07 and 85.18 % relative activity of that of the parental strain *Aspergillus* sp. NR463 (which was taken as 100%). *Aspergillus* sp. NR463U4 with sufficiently high stability was the strain of choice due to its highest myrosinase production compared to other mutants at the same culture volume. *Aspergillus* sp. NR463U4 growing in mustard extract medium with no supplement gave relatively high myrosinase activity (2.35 units ml^{-1}) compared with those from previous work; *Aspergillus syndowi* IFO4284 (0.08 units ml^{-1}) (Ohtsuru *et al.*, 1969), *Aspergillus niger* AKU3302 (0.04 units ml^{-1}) (Ohtsuru and Hata, 1973), *Aspergillus syndowi* QM31c (0.097 units ml^{-1}) (Petroski and Kwolek, 1985) and *Aspergillus* sp. NR-4201 (0.875 units ml^{-1}) (Sakorn *et al.*, 1999). To our knowledge, myrosinase-overproducing mutants characterized by such high levels of enzymatic activities have not been previously reported. The advantages of using *Aspergillus* sp. NR463 and its mutants are their higher myrosinase activity and the ability to grow in low cost media.

The stability of the enzymes present in the cell free extracts was subsequently determined. Myrosinase from the wild-type *Aspergillus* sp. NR463 was not fully stable such that it was inactive after 2 hr at 30°C. The stability of the wild-type enzyme was similar to those of enzymes from other microorganisms suggesting that the myrosinase of microorganisms were not stable at high temperatures (Lazzeri *et al.*, 1993; Petruccioli *et al.*, 1995). UV mutagenesis was able to increase myrosinase stability. Even though the enzymes of fungal strain NR463U5 lost stability after 4 hr, those of strains NR463U1, NR463U2, NR463U3 and NR463U4 remained stable for nearly 7 hr at 30°C, which was 3.5 times longer than the wild-type strain *Aspergillus* sp. NR463. The myrosinase from the mutants reported here remained stable at 4°C for 2 weeks (data not shown). Sakorn *et al.* (1999) reported that myrosinase from *Aspergillus* sp. NR-4201 was stable for several days at 4°C and Ohtsuru *et al.* (1973) reported that myrosinase from *Aspergillus niger* AKU3302 had low stability when stored at 5°C. The product analysis of myrosinase from wild-type *Aspergillus* sp. NR463 and mutant strains (NR463U1, NR463U2, NR463U3, NR463U4 and NR463U5) revealed that glucosinolate was degraded to allylisothiocyanate in neutral conditions, while allylcyanide could not be detected. The main glucosinolate found in mustard seed cake (*Brassica juncea* var. Forge) was sinigrin. This result was similar to other previous reports. Palop *et al.* (1995) reported that myrosinase from *Lactobacillus agilis* R16 was involved in sinigrin hydrolysis, resulting in the

production of allylisothiocynate and glucose. Smiths *et al.* (1993) reported that at pH 7.2 allylisothiocyanate and very low amounts of allylcyanide were produced by cell-free extracts of *Aspergillus calvatus* II9. In addition, Sakorn *et al.* (1999) found that myrosinase from *Aspergillus* sp. NR-4201 was active at neutral pH and allylisothiocyanate was the main product. The types of products obtained from glucosinolate degradation are depended on the source, type of glucosinolate and the pH levels of enzyme reactions. This result was useful for allylisothiocyanate production and other products from glucosionolate hydrolysis.

The growth profile of mutant strains suggested that all the mutants gave the same time course of growth and optimal condition as the wild-type strain. The results showed the time course of growth and enzyme activity of mutant strain *Aspergillus* sp. NR463U4 cultivated under optimal culturing conditions (30°C, pH 6.5). Maximum myrosinase activity was reached after 36 hr of growth. Glucosinolate was degraded completely in 36 hr of growth. This growth profile was better than previous work on the biodegradation by *Aspergillus* sp.NR-4201 at 5.5-6 mM of glucosinolate (Sakorn *et al.*, 1999; Rakariyatham and Sakorn, 2002). The mutant strains degraded higher glucosinolate concentrations (10 mM) at the same time course of growth in liquid media. Glucosinolate concentration also decreased rapidly in the beginning of the cultivation. The growth profile had small amounts of glucose liberated to the culture media. In this case, glucose should be liberated from some other polysaccharides, not

the glucosinolate. It was previously proposed that *Brassica* oilseeds contain variable amounts of polysaccharides. Rakariyatham (2000) has confirmed that mustard seed meal (*Brassica juncea* var. Forge) contained 21.3% carbohydrates. These polysaccharides may serve as substrates of glucoamylase from wild-type and mutant strains observed in this study by a release of glucose into the culture medium. Production stability of the mutant strain was studied. Cultures were transferred every 15 days and tested monthly for 8 months for the presence of myrosinase activity.

Myrosinase production by the mutant strains showed only a small variation for, at least, 16 slant-to-slant transfers. This result demonstrated that wild-type and mutant strains of *Aspergillus* sp. NR463 have a small variation in myrosinase production after long storage time.

4.1.4 Chemical mutagenesis of *Aspergillus* sp. NR463

The kinetics of mutagenesis of *Aspergillus* sp. NR463 was carried out at different time intervals and the survival percentage of *Aspergillus* sp. NR463 showed 10 % survival rate of spores during 130 min incubation, after which the rapid killing of spores was observed and at 160 min of incubation no spores survived. By screening the survived spores, four of each chemical mutant, NR463E1-E5 (EMS) and NR463MG1-MG5 (MNNG) were selected on the basis of myrosinase production.

The result of mutagenesis gave 10 candidate mutant strains. The mutant strains NR463E1, NR463E2, NR463E3, NR463E4, NR463E5, NR463MG1, NR463MG2, NR463MG3, NR463MG4 and NR463MG5 had activities of 1.576, 1.682, 1.810, 1.320, 1.350, 1.888, 2.017, 1.637, 1.395 and 1.335 units ml^{-1} respectively, which had 115, 123, 132, 96, 98, 139.85, 149.41, 121, 104.5 and 100.5% relative activity of that of the parental strain *Aspergillus* sp. NR463 (which was taken as 100%). Strains NR463E2, NR463E3, NR463MG1 and NR463MG3 with sufficiently high stability were the strains of choice due to their high myrosinase production compared to other mutants at the same culture volume. Strains NR463E2, NR463E3, NR463MG1 and NR463MG3 grown on mustard extract medium with no supplement gave relatively high myrosinase activity (1.682, 1.810, 1.888 and 1.637 units ml^{-1} respectively) compared with those from previous work: *Aspergillus syndowi* IFO4284, 0.08 units ml^{-1} (Ohtsuru *et al*., 1969); *Aspergillus niger* AKU3302, 0.04 units ml^{-1} (Ohtsuru and Hata, 1973); *Aspergillus syndowi* QM31c, 0.097 units ml^{-1} (Petroski and Kwolek, 1985); and *Aspergillus* sp. NR-4201, 0.875 units ml^{-1} (Sakorn *et al*., 1999). To our knowledge, myrosinase overproducing chemical mutants characterized by such high levels of enzymatic activities have not been previously reported. The advantages of using *Aspergillus* sp. NR463 and its mutants were the possession of higher myrosinase activity and the ability to grow in the low cost medium (mustard extract medium with no supplement).

The stability of enzymes present in the cell-free extracts was subsequently determined. Chemical mutagenesis was able to increase myrosinase production in NR463E2, NR463E3, NR463MG1 and NR463MG3 strains and myrosinase stability was more stable than the wild-type. The myrosinase from all mutants reported here remained stable at 4°C for 11 weeks (data not shown). Ohtsuru *et al.* (1973) reported that myrosinase from *Aspergillus niger* AKU3302 had low stability when stored at 5 °C and Sakorn *et al.* (1999) reported that myrosinase from *Aspergillus* sp. NR-4201 was stable for several days at 4°C.

The growth profile of mutant strains suggested that all the mutants gave the same time course of growth at optimal condition as the wild-type strain. Maximum myrosinase activity was reached after 36 hr of growth. Glucosinolate was degraded completely in 36 hr of growth. This growth profile was better than previous work on the biodegradation by *Aspergillus* sp. NR-4201 at 5.5-6 mM of glucosinolate (Sakorn *et al.*, 1999; Rakariyatham and Sakorn, 2002). The mutant strains NR463E1, NR463E2, NR463MG1 and NR463MG3 degraded high glucosinolate concentration (10 mM) at the same time course of growth in liquid media. Glucosinolate concentration also decreased rapidly in the beginning of the cultivation.

4.1.5 RAPD analysis of *Aspergillus* sp. NR463 mutant strains

To verify that the mutant isolates obtained were isogenic variants of the wild-type strains and not accidental contaminants, RAPD fingerprinting was used. The RAPD profiles were obtained with the selected primer (OPN02, OPN04, OPN05, OPN06, OPN07, OPN11, OPN12, OPN13, OPN14 and OPN16). This technique was capable to readily distinguish between the 7 isolates (NR463U2, NR463U3, NR463U4, NR463E2, NR463E3, NR463MG1 and NR463MG3) compared with wild-type. The sensitivity of the technique extended to the strain level, as 7 *Aspergillus* sp. NR463 strains were readily distinguished. All of the 7 selected strains have genome different from wild-type.

4.1.6 Analysis of glucosinolate breakdown products by gas chromatography

The product analysis of myrosinase from wild-type *Aspergillus* sp. NR463 and mutant strains (NR463U2, NR463U3, NR463U4, NR463E2, NR463E3, NR463MG1 and NR4617MG3) revealed that glucosinolate was degraded to allylisothiocyanate in neutral condition, while allylcyanide could not be detected. The main glucosinolate found in mustard seed cake (*Brassica juncea* var. Forge) was sinigrin. This result was similar to other previous reports. Palop *et al.* (1995) reported that myrosinase from *Lactobacillus agilis* R16 was involved in sinigrin hydrolysis, resulting in the

production of allylisothiocynate and glucose. Smiths *et al.* (1993) reported that at pH 7.2 allylisothiocyanate and very low amounts of allylcyanide were produced by cell-free extracts of *Aspergillus calvatus* II9. In addition, Sakorn *et al.* (1999) found that myrosinase from *Aspergillus* sp. NR-4201 was active at neutral pH levels and allylisothiocyanate was a main product. The types of products obtained from glucosinolate degradation are depending on the source and type of glucosinolate and the pH of the enzyme reaction. This result was useful for allylisothiocyanate production and other products from glucosionolate hydrolysis.

4.1.7 Kinetic constants

There were some similar properties between the *Aspergillus* sp. NR463 myrosinase and the other three myrosinases from *Aspergillus* sp. NR-4201 (Sakorn *et al.*, 1999), *A. syndowi* IFO4284 (Ohtsuru *et al.*, 1969) and *A. niger* AKU3302 (Ohtsuru *et al.*, 1973) (Table 4.2). Firstly, their pH optima were nearly neutral as like *Aspergillus* sp. NR4201, while, those of the other plant enzymes were acidic ranging from 5.5-6.5 (Bones and Rossiter, 1996). Secondly, their temperature optima were relatively low (25-37°C) but plant enzymes, exemplified by *Sinapis alba* in Table 4.2, were present at higher temperatures of about 55-65°C. Thirdly, the stability of all microbial enzymes was temperature dependent.

Table 4.2 Comparison of physico-chemical properties of myrosinase from *Aspergillus* sp. NR463, *Aspergillus* sp. NR-4201 (Sakorn *et al.*, 1999), *A. syndowi* IFO4284 (Ohtsuru *et al.*, 1969), *A. niger* AKU3302 (Ohtsuru and Hata, 1973) and *Sinapis alba* (Bjorkman and Janson, 1972; Bjorkman and Lonnerdal, 1973).

Property	NR463	NR-4201	IFO4284	AKU3302	*S. alba*
Optimal temperature (°C)	30	28	25	34	60
Optimal pH	7.8-8.0	7.4	7	6.2	6.5
Molecular mass (kDa)	ND	94	120	90	135
Km for sinigrin (mM)	0.82	0.645	3.6	3.3	0.17

ND = not detected.

In this study, K_m values for sinigrin of the crude *Aspergillus* sp. NR463 and of *Aspergillus* sp. NR463U4 were the same. The K_m determination by the spectrophotometric assay (0.25 mM) was about 3.28 times less than that determined by the coupled enzyme assay (0.82 mM). It was generally accepted that the K_m value assessed at higher substrate concentrations was more reliable. Regarding the affinity of the *Aspergillus* enzyme to sinigrin, the K_m value for sinigrin hydrolysis (0.82 mM) was approximately 4.39 times less than those values of the *A. syndowi* (3.6 mM) and *A. niger* (3.4 mM) enzymes (Ohtsuru *et al.*, 1969; Ohtsuru and Hata, 1973). It was considered that the affinity of plant myrosinase to glucosinolate substrates was about twenty times higher than that for pNPG (Ohtsuru *et al.*, 1969). Alternatively, the affinity of microbial enzymes for pNPG was higher than that for glucosinolates (Ohtsuru *et al.*, 1969). The results indicated that the affinity of the *Aspergillus* sp.

NR463 myrosinase for glucosinolate, like sinigrin, was between those of plant enzymes (Bone and Rossiter, 1996) and those of two fungal enzymes previously reported (Ohtsuru *et al.*, 1969; Ohtsuru and Hata, 1973).

4.2 Conclusion

Twenty seven fungal isolates, a *Rhizopus* sp., a *Mucor* sp. and 25 *Aspergillus* sp., and 11 gram negative rod bacterium were obtained from soil by growing in sinigrin agar plates. All microbial strains were assessed for glucosinolate degradative potential in liquid culture by using sinigrin as a model substrate. Sinigrin degrading activity was shown by three fungal strains but not by the bacterium. Although there were three fungi, only the 21 strains in the genus *Aspergillus* exhibited potential for degrading sinigrin. The fungal strains were identification and also confirmed by MIRCEN (UNESCO World Network of Microbiological Resources Centre), Bangkok Thailand, where it was stored. Preliminary studies of 21 *Aspergillus* strains were found to be different in colony morphologies and intracellular protein patterns. The most effective myrosinase producer was *Aspergillus* sp. NR463. The NR463 strain also exhibited a high potential to degrade glucosinolate which were contained in brown mustard seeds (*Brassica juncea*) and also produced high myrosinase activity.

For production of myrosinase by *Aspergillus* sp. NR463, cultivation was performed by one step liquid culture. Optimum conditions for enzyme production

were achieved in mustard extract medium with glucosinolate concentration of 10 mM, operating at pH 6.5 and temperature of 30°C for 48 hr, under reciprocal shaking at 150 rpm. Cell free extracts obtained after fungal cell disruption of a 50 ml culture contained 68.45 units of myrosinase activity (1.369 units per ml of the culture medium).

Physical and chemical mutagenesis was done using UV, ethylmethane sulfonate (EMS) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) to treat spores at the appropriated times. The results presented 7 strains (NR463U2, NR463U3, NR463U4, NR463E2, NR463E3, NR463MG1 and NR463MG3) that have myrosinase overproduction and a prolonged stability greater than the wild-type. The selected mutant strains were examined by RAPD to distinguish the 7 isolates by using 10 primers. The result was clearly illustrated that all the mutants have different genome from the wild-type strain. All of the mutant strains had the same optimal conditions of the wild-type. Production stability of the mutant strains was also investigated for myrosinase production and mutation variation. The results showed that all of mutant strains produced myrosinase in low variations. The most effective mutant strain was *Aspergillus* sp. NR463U4. Cell-free extracts obtained after fungal cell disruption of a 50 ml culture contained 117.5 U of myrosinase activity (2.35 units per ml of the culture medium). This is the higest myrosinase production ever reported by a microorganism. The enhancement of myrosinase activity might be due to structural

change of the enzyme, higher amount of the enzyme, increased level of modulator proteins or cofactors promoting the enzyme activity or decreased level of inhibitory proteins.

Optimum activity of the enzyme was shown as the same condition of the wild-type at pH 8.0 and at temperature of 29-30°C. Enzyme kinetics of crude myrosinase was shown as K_m and V_{max} with sinigrin by spectrophotometric method calculated to be 0.25 mM and 28.86 μmol $min^{-1}mg^{-1}$protein and couple enzyme method calculated to be 0.82 mM and 95 μmol $min^{-1}mg^{-1}$protein respectively. Glucosinolate hydrolysis by crude myrosinase at pH 7.5, yielded allylisothiocyanate as a main product. Whereas, no liberation of allylcyanide was observed in the reaction mixtures.