Sinigrin Degradation by *Aspergillus* sp. NR-4201 in Liquid Culture

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ABSTRACT A myrosinase-producing fungus, *Aspergillus* sp. NR-4201, was newly isolated from decayed mustard seed meal samples obtained in Lamphun, Thailand. When preincubated in a medium containing sinigrin, myrosinase was expressed intracellularly whereas none was detected in sinigrin-free medium. Sinigrin degradation was closely related to the presence of myrosinase. Induced mycelium consumed both glucose and sinigrin competitively, while non-induced myceliun exhausted glucose first and then sinigrin, with no myrosinase being produced during the glucose consumption period. The product allylcyanide was detected in incubation mixtures but its accumulation was delayed. Cell-free extracts incubated with sinigrin produced allylisothiocyanate at pH 5.6 and 7.2 but not at pH 4.0.

KEYWORDS: sinigrin, glucosinolates, myrosinase, *Aspergillus*.

INTRODUCTION

Glucosinolates are a group of thioglucoside compounds that occur exclusively as vacuolar granules in non-specific cells of *Crucifers*.[1] Sinigrin (allylglucosinolate or 2-propenylglucosinolate) is a major glucosinolate located especially in brown mustard seeds (*Brassica juncea*) used as raw materials for industrial production of mustard volatile oil, allylisothiocyanate (AIT). In addition to glucosinolates, the enzyme myrosinase (thioglucoside glucohydrolase or thioglucosidase, EC 3.2.3.1) is contained separatedly as myrosin grains in specific myrosin cells.[1-2] Upon tissue disruption, the stored glucosinolates are exposed to this degradative enzyme which hydrolytically cleaves the thioglucoside linkage to yield D-glucose and an unstable thiohydroxymate-O-sulfonate intermediate. The intermediate undergoes spontaneous rearrangement, resulting in production of sulfate and one of four possible reaction products. These are nitrile, isothiocyanate, thiocyanate or cyanoepithioalkane depending on substrate, pH or availability of ferrous ion and epithiospecifier protein (ESP) as shown in Fig 1.

Brassica oilseeds rank fifth in seed oil production of the world.[3] The oilseed meals remaining after oil production are enriched in proteins and very well-balanced amino acid composition which can be used in animal feeds.[4] However, the usefulness of these meals is restricted by their variable content of antinutritional glucosinolates. Although intact glucosinolates are non-toxic, their hydrolytic products are toxic to experimental animals producing such problems as thyroid hypertrophy, liver haemorrhage, growth retardation, etc.[5-6] Destruction of seed myrosinase before consumption cannot prevent these effects since a number of gastrointestinal microflora tract express myrosinase or myrosinase like activity.[7-8] Several physico-chemical methods have been attempted to eliminate these undesirable substances but none have been applied practically.[9-11] Recently, research on nutritional improvements of *Brassica* oilseed meal have focussed on biological processing. A large number of microorganisms such as *Escherichia coli*,[5] *Lactobacillus acidophilus*,[6] *Bacillus cereus*,[12] *Enterobacter cloacae*[13] *and Aspergillus niger*[14] have been reported to have sinigrin or progoitrin degradating activity. However, their degradative potential seemed to be low. A bacterium *Lactobacillus agilis* R16[15] and two fungal strains, *Aspergillus clavatus* II-9 and *Fusarium oxysporum* @146[16] were characterized and proved to have high degradative potential of pure sinigrin and glucosinolates in mustard meal.

Our work describes the overall characterization of a new isolate of *Aspergillus* sp. for its potential for sinigrin degradation. This is closely related to its intracellular myrosinase activity.

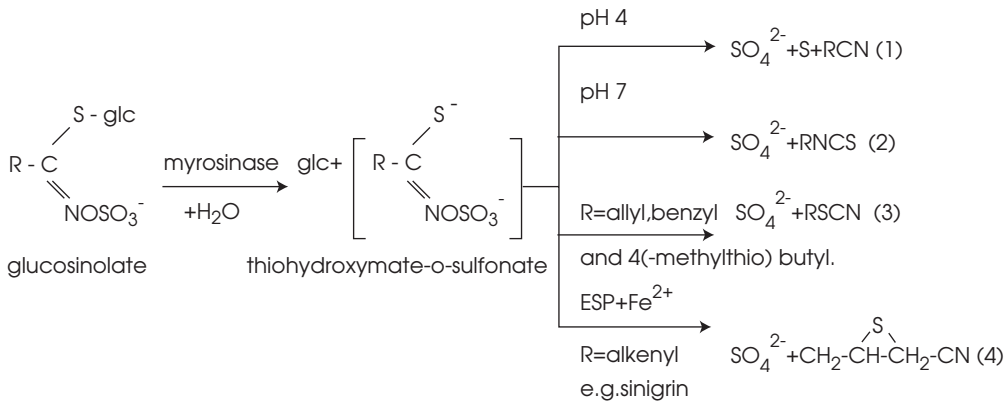

Fig 1. The myrosinase catalyzed degradation of glucosinolates to yield (1) nitriles (2) isothiocyanates(3) thiocyanates and (4) cyanoepithioalkanes. (derived from reference no.1)

Materials and Methods

Fungal strains

Quantities of 0.5 g of each decayed mustard seed meal sample (10 samples collected from Lamphun, Thailand) were suspended in 25 ml sterile distilled water and allow to settle for 30 min. Then, 100 µl of supernatant were spread onto sinigrin agar plates (0.1 M potassium phosphate buffer, pH 5.6 containing 5 mM sinigrin, 6.7 g/l yeast nitrogen base and 10 g/l agar) and incubated for 7 days at room temperature. Each distinct isolate was plated on Czapek Dox agar and subcultured until pure colonies were observed. Selected isolates were maintained on potato dextrose agar slants and subcultured monthly. For preliminary screening of sinigrin degradation potential, spore suspensions were inoculated in 2.5 ml of sinigrin-glucose medium (0.1 M potassium phosphate buffer, pH 5.6 containing 5.5 mM sinigrin, 5.5 mM glucose and 6.7 g/l yeast nitrogen base). After incubation at 28°C in a waterbath shaker at 150 rpm, the concentrations of sinigrin and glucose were determined daily.

Inoculum

Spore suspensions for inocula were obtained from 2-week-old-cultures on potato dextrose agar slants by adding sterile 0.1 M potassium phosphate buffer, pH 5.6. The suspensions were vigorously shaken and then adjusted to a concentration of 10^6 spores/ml using a Neubauer haemocytometer.

Resting cell experiments

Non-induced cells used for incubation experiments were cultivated by inoculating 25 µl of spore suspension into 2.5 ml of growth medium (0.1 M potassium phosphate buffer, pH 5.6 containing 11 mM glucose and 6.7 g/l yeast nitrogen base) contained in 25-ml erlenmeyer flasks and incubated at 28°C in a waterbath shaker at 150 rpm. Induced cells were obtained with the same procedure, but using the induction medium (0.1 M potassium phosphate buffer, pH 5.6 containing 10 mM glucose, 1 mM sinigrin and 6.7 g/l yeast nitrogen base) was used. After 32 h incubation, cells were harvested by centrifugation (5,000 xg, 10 min) and washed twice with sterile 0.1 M potassium phosphate buffer, pH 5.6. Cell pellets were suspended in 2.5 ml of 0.1 M potassium phosphate buffer, pH 5.6 containing 5.5 mM sinigrin (non-growth condition) or 0.1 M potassium phosphate buffer, pH 5.6 containing 5.5 mM glucose, 5.5 mM sinigrin and 6.7 g/l yeast nitrogen base (growth condition) and then incubated as above. During incubation, samples were taken periodically by centrifugation (5,000xg, 10 min). Cell-free supernatants were analyzed for glucose, sinigrin, allylcyanide, allylisothiocyanate and myrosinase contents. After washing twice with 0.05 M phosphate buffer, pH 7.2 and adding 0.5 ml of 0.05 M phosphate buffer, pH 7.2, fungal cell pellets were disrupted immediately by mortar at 4°C and then centrifuged (10,000xg, 10 min, 4°C). The cell-free extracts were adjusted to 1.0 ml with 0.05 M phosphate buffer, pH 7.2 and then assayed for intracellular myrosinase activity.

Cell-free extract experiments

Cell-free extracts used in these experiments were prepared from fungal cells grown in sinigrin-glucose medium for 36 h as described above. Washing and disrupting of the cells were performed with distilled water at 4°C. Quantities of 800 µl of 0.1 M of three

designed buffers (citrate buffer pH 4.0, phosphate buffer pH5.6 and phosphate buffer pH 7.2) containing 5 mM sinigrin and 200 µl of cell-free extracts were mixed and incubated at room temperature. Samples from incubation mixtures were taken periodically and assayed for glucose, sinigrin, allylcyanide and allylisothiocyanate concentrations.

Analyses

Extracellular myrosinase activity in cell-free supernatants collected during incubation experiments was measured by the method described by Palmieri *et al*.[17] One ml of potassium phosphate buffer, pH 5.6 containing 0.1 M sinigrin and 100 µl of sample were mixed gently and measured at 227.5 nm using a double beam spectrophotometer (UV/VIS Hitachi U 2000). Enzyme activity was calculated from the decrease in absorbance with time ($\varepsilon_{227.5\ nm}$ for sinigrin = 6950 M^{-1} cm^{-1}).

Myrosinase activity in cell-free extracts was kinetically measured by a method based on a coupled enzyme assay with a slight modification.[18] After preincubation at 28°C, a 50 µl sample and 1 ml of reagent (30 mM morpholinoethane sulfonic acid buffer, pH 7.2 containing 3 mM $MgCl_2$, 0.55 mM ATP, 0.72 mM NADP, 3.5 U hexokinase and 1.75 U glucose-6- phosphate dehydrogenase) were mixed and then monitored at 340 nm. Myrosinase activity was calculated from the rate of absorbance change over time due to the formation of NADPH (ε_{340nm} for NADPH = 6220 M^{-1} cm^{-1}). One unit of myrosinase was defined as the amount of enzyme which catalyzed the liberation of 1 µmol of glucose per minute from sinigrin under the conditions described above.

Sinigrin (glucosinolates) and glucose levels were quantified by the coupled enzyme method.[18] A sample or standard solution (50 µl) was added to 1 ml of reaction mixture (30 mM morpholinoethane sulfonic acid buffer, pH 6.5 containing 3 mM $MgCl_2$, 1 mM ascorbic acid, 0.55 mM ATP and 0.72 mM ATP). After mixing, the absorbance at 340 nm was read (E_1). Then, 5 ml of hexokinase/glucose-6-phosphate dehydrogenase mixture (activities of 700 and 350 U/ml, respectively) was added and incubated for 15 min at 30°C. The second absorbance (E_2) was then measured. For quantification of sinigrin, 0.14 U of commercial myrosinase was added to the reaction mixture in order to liberate free glucose from sinigrin. After additional incubation for 30 min at 30 °C, the third absorbance (E_3) was read. Glucose content could be calculated from E_2 - E_1 by using calibration curves for glucose. Sinigrin level was determined from the absorbance difference, E_3 - E_2, using the same calibration curve.

Allylcyanide content was determined by GC employing a Shimadsu 14A gas chromatograph (Shimadsu, Japan) equipped with a flame ionization detector (FID) and a Chromatopack column (50m x 0.30mm ID) connected to the injector port. The carrier gas (helium) was constantly operated at a flow rate of 0.5 cm^3/min and a pressure of 50 kPa. Temperatures of the column, injector and detector were maintained at 80, 230 and 280°C, respectively. One ml of sample or standard solution was extracted with 2 ml of hexane (GC grade). After adding approximately 2 g of solid ammonium sulfate, sealing, shaking (5 min) and centrifuging (3,000xg, 10 min), 1 µl of hexane layer was injected.

GC analysis of allylisothiocyanate was done following the procedure described above but the flow rate of carrier gas was adjusted to 1.0 cm^3/min and the column temperature was programmed to increased 10°C/min from the initial temperature of 80°C.

Chemicals

Sinigrin monohydrate, myrosinase (thioglucosidase, EC 3.2.3.1), allylcyanide, allylisothiocyanate and morpholinoethane sulfonic acid were purchased from Sigma (USA). NADP, ATP. and hexokinase/glucose-6-phosphate dehydrogenase were from Boehringer Mannheim (FRG). Yeast nitrogen base, potato dextrose agar, peptone and agar were provided from Difco (USA). All chemicals used in experiments were analytical grade.

Results

Screening

After incubation for 7 days, 4 fungal isolates were able to grow on sinigrin agar plates. However, only one of them consumed sinigrin from sinigrin-glucose media completely within 3 days and it was identified according to Raper and Fennell[19] as *Aspergillus* sp. This *Aspergillus* strain was designated NR-4201 and used throughout the experiments described here in.

Resting cell experiments

Preincubation growth of the *Aspergillus* sp. NR-4201 in a medium containing sinigrin resulted in expression of approximately 0.10 U/ml of intracellular myrosinase activity (Fig 2). While cultured in sinigrin-free medium, no myrosinase activity was detected. Sinigrin consumption started immediately when induced cells were incubated with sinigrin (Fig 2B). Then, levels of intracellular myrosinase activity increased rapidly from 0.10 to 0.28 U/ml within 3 h

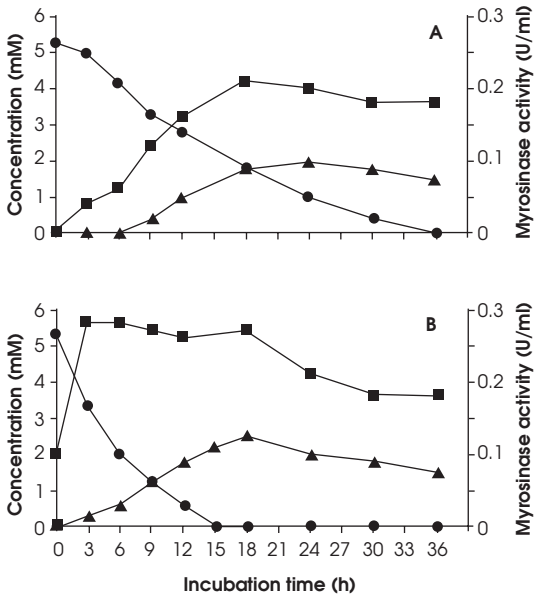

Fig 2. Degradation of sinigrin (●), formation of allylcyanide (▲) and occurrence of intracellular myrosinase activity (■) by non-induced cells (A) and induced cells (B) of *Aspergillus* sp. NR-4201 under non-growth conditions.

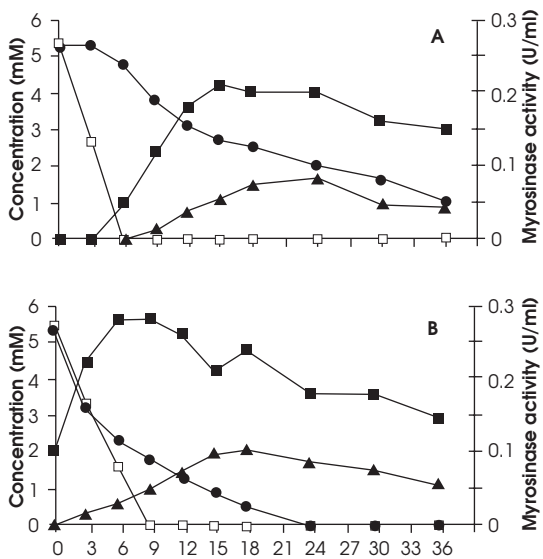

Fig 3. Degradation of sinigrin (●), and glucose (❑), formation of allylcyanide (▲) and occurrence of intracellular myrosinase activity (■) by non-induced cells (A) and induced cells (B) of *Aspergillus* sp. NR-4201 under growth conditions.

and remained constant for at least 18 h before declining. Incubation of non-induced cells with sinigrin resulted in progressive expression of intracellular myrosinase upto maximum levels (~0.2 U/ml) within 18 h (Fig 2A). The non-induced cells gave total sinigrin degradation in 36 h, compared with 15 h using induced cells. Accumulation of the product, allylcyanide, started at 9 and 3 h, respectively, for the non-induced and induced cells. The maximum levels of allylcyanide formed were approximately 38 and 40% of the initial sinigrin concentrations, respectively, for the non-induced and induced cells. However, free glucose, myrosinase activity and allylisothiocyanate were not detected in cell-free supernatants in either experiment.

When cells were grown in sinigrin medium supplemented with glucose, the result was quite different (Fig 3). Glucose was totally consumed within 6 h by the non-induced cells (Fig 3A) and during the first 3 h of incubation, they did not degrade sinigrin and had no myrosinase activity. After glucose was exhausted, myrosinase was produced and sinigrin was consequently degraded. However, the total amount of sinigrin available was not consumed within 36 h. Sinigrin and glucose were competitively used by induced cells of the *Aspergillus* (Fig 3B). However, the cells used up glucose faster than sinigrin. Maximum myrosinase activity was reached within 6 h and total degradation was achieved in 24 h. The maximum levels of allylcyanide production were 30 and 35% of the initial sinigrin concentrations, respectively for the non-induced and induced experiments. No extracellular accumulation of allylisothiocyanate or myrosinase activity was detected, as in the non-growth experiments.

Cell-free extract experiments

Incubation of cell-free extracts of the *Aspergillus* sp. with sinigrin at pH 7.2 and 5.6 resulted in production of glucose which was stoichiometric to the amount of sinigrin degraded. At pH 7.2, sinigrin was completely degraded within 180 min whereas sinigrin degradation stopped after 120 min at pH 5.6. Allylcyanide was not produced in these experiments, while allylisothiocyanate was non-stoichiometrically formed at pH 5.6 and 7.2. No sinigrin was degraded and no product was formed at pH 4.0.

Discussion

The *Aspergillus* sp. isolated in our laboratory demonstrated high potential of sinigrin degradation

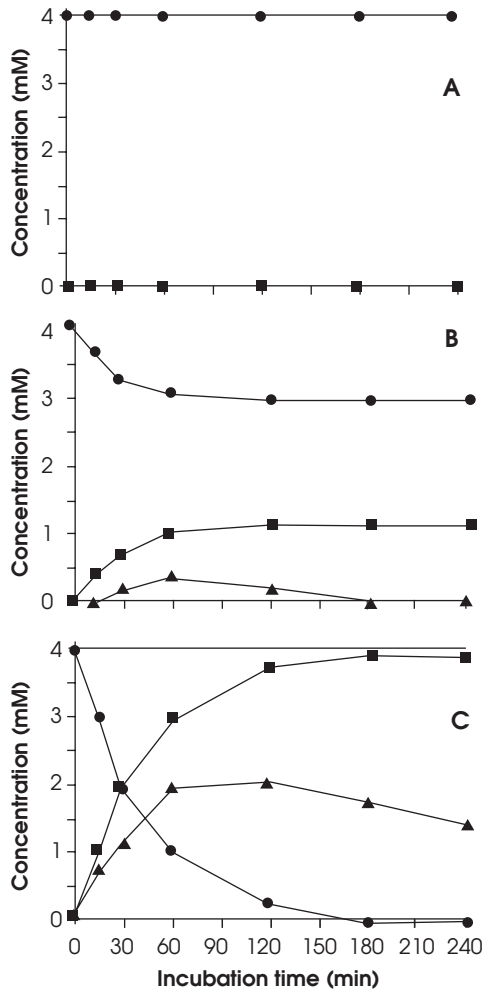

Fig 4. Incubation of cell-free extract of *Aspergillus* sp. NR-4201 with sinigrin at pH 4.0 (A), pH 5.6 (B) and pH 7.2 (C). Degradation of sinigrin (●), production of glucose (■) and allylisothiocyanate (▲) are shown.

in liquid cultivation. The capability of the fungus to consume sinigrin depended on available myrosinase activity. There are some interesting points from the resting cell experiments. First, maximum myrosinase activities were significantly higher in induced cells. Second, incubation times required to reach maximum myrosinase activity was shorter for the induced cells, eventually non-induced cells could degrade sinigrin extensively. Third, even after sinigrin was exhausted, the enzyme level remained high for several hours. After cell disruption, myrosinase from cell-free extracts was stable for several days at 4°C (data not shown). By contrast, Ohtsuru *et al*.[14] reported that myrosinase from *Aspergillus niger* AKU3302 had low stability when stored at 5°C.

Incubation of whole cells of the *Aspergillus* in a medium containing sinigrin yielded allylcyanide. This was similar to the result with *Aspergillus clavatus* II-9, reported by Smiths *et al*.[16] However, the maximum levels of allylcyanide formed by the *A. clavatus* (68 and 50% of the initial concentration of sinigrin under growth and non-growth conditions, respectively) were higher than those seen in our experiments.

The fact that our induced cells could degrade both glucose and sinigrin at the same time different from two other fungal strains,*Aspergillus clavatus* II-9 and *Fusarium oxysporum* @149.[16] Induced cells of *A. clavatus* consumed sinigrin completely and then glucose whereas those of *F.oxysporum* degraded glucose exhaustively and then sinigrin. Induced cells of the bacterium, *Lactobacillus agilis* R16, also consumed glucose prior to sinigrin[15]

It was surprising that allylisothiocyanate rather than allylcyanide was observed in incubation mixtures of cell-free extracts and sinigrin at pH 5.6 and 7.2. Smiths *et al* [16] reported that allylisothiocyandte and very low amounts of allylcyanide were produced by cell-free extracts of *A.clavatus* and at pH 6.2. Gil and Macleod[20] established that mustard seed myrosinase gave allylcyanide at pH 4 and allylisothiocyanate at pH 7 and the optimum pH for most plant myrosinases ranges from 4.5-5.5.[1] By contrast, myrosinase from the *Aspergillus* sp. NR-4201 did not function at pH 4 and is most active at neutral pH. Increasing acidity in assay mixtures resulted in decreased enzyme activity (unpublished data).

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References

1. Bones AM and Rossiter JT (1996) The myrosinase-glucosinolate system, its organisation and biochemistry. *Physiol Plant* **97**, 194-208.
2. Hoglund AS, Lenman M and Rask L (1992) Myrosinase is localized to the interior of myrosin grains and is not associated to the surrounding tonoplast membrane. *Plant Sci* **85**, 165-70.
3. Maheshwari PN, Stanley DW and Gray JI (1981) Detoxification of rapeseed products. *J Food Protect* **44(6)**, 459-70.
4. Miller RW, VanEtten CH, McGrew C and Wolff IA (1962) Amino acid composition of seed meals from forty-one species of Cruiferae. *J Agr Food Chem* **10**, 426-30.
5. Wight PAL, Scougall RK, Shannon DWF and Wells JW (1987) Role of glucosinolates in the causation of liver haemorrhages in laying hens fed water-extracted or heated-treated rapeseed-cakes. *Res Vet Sci* **43**, 313-9.

6. Fenwick OR and Heaney RK (1983) Glucosinolates and their breakdown products in cruciferous crops, foods and feedingstuffs. *Food Chem* **11**, 249-71.
7. Oginsky EL, Stein AE and Greer MA (1965) Myrosinase activity in bacteria as demonstrated by the conversion of progoitrin to goitrin. *Pro Soc Exp Med* **119**, 360-4.
8. Nugon-Baudon L, Rabot S, Wal JP and Szylit O (1990) Interactions of the intestinal microflora with glucosinolates in rapeseed meal toxicity: first evidence of an intestinal *Lactobacillus* possessing a myrosinase-like activity *in vivo*. *J Sci Food Agri* **52**, 547-59.
9. Kozlowska H, Sosuski FW and Youngs CG (1972) Extraction of glucosinolates from rapeseed. *Can Int Food Sci Technol J* **5**, 149-54.
10. Sosulski FW, Soliman FS and Bhatty RS (1972) Diffusion extraction of glucosinolates from rapeseed. *Can Int Food Sci Technol J* **5**, 101-4.
11. Shahidi F and Gabon JE (1990) Fate of sinigrin in methanol/ammonia/water-hexane extraction of *B. juncea* mustard seed. *J Food Sci* **55(3)**, 793-5.
12. Huber J, Kranz G, Kreibich G, Beining K, Kruger M and Weissbach F (1983) Mikrobieller abbau von glucosinolaten in rapsectraktionsschrot. *Die Nahrung* **27**, 257-63.
13. Tani N, Ohtsuru M and Hata T (1974) Isolation of myrosinase producing microorganism. *Agri Biol Chem* **38(9)**, 1617-22.
14. Ohtsuru M, Tsuruo I and Hata T (1973) The production and stability of intracellular myrosinase from *Aspergillus niger*. *Agri Biol Chem* **37(5)**, 967-71.
15. Palop ML, Smiths, JP and ten Brink, B (1995) Degradation of sinigrin by *Lactobacillus agilis* strain R16. *Int J Food Microbiol* **26**, 219-29.
16. Smiths JP, Knol W and Bol J (1993) Glucosinolate degradation by *Aspergillus clavatus* and *Fusarium oxysporum* in liquid and solid-state fermentation. *Appl Microbiol Biotechnol* **38**, 696-701.
17. Palmieri S, Leoni O and Iori R (1982) A steady-state kinetic study of myrosinase with direct ultraviolet spectrophotometric assay. *Anal Biochem* **123**, 320-4.
18. Wilkinson AP, Rhodes MJC and Fenwick GR (1984) Determination of myrosinase (thioglucoside glucohydrolase) activity by a spectrophotometric coupled enzyme assay. *Anal Biochem* **139**, 284-91.
19. Raper KB and Fennell DI (1965) The genus *Aspergillus*. *Baltimore: The Williams & Wilkins Co*.
20. Gil V and MacLeod AJ (1980) The effects of pH on glucosinolate degradation by a thioglucoside glucohydrolase preparation. *Phytochem* **19**, 2547-51.